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(54) Title: COMBINATION FOR REDUCING ANTIMICROBIAL RESISTANCE USING A METHYLATION INHIBITOR IN COMBINATION WITH AN ANTIBIOTIC

(57) Abstract

Disclosed are novel methods, combinations of agents and kits for use in killing, or inhibiting the growth of microorganisms. Enhanced antimicrobial action is provided by using a methylation inhibitor, as exemplified by using an agent that inhibits methylation or maturation of bacterial RNA in combination with, e.g., a macrolide lincosamide streptogramin B (MLS) antibiotic. The methods and compositions described may be employed to reduce the resistance of susceptible microorganisms to antimicrobial agents and thus to treat animals or patients with infections.

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## DESCRIPTION

5 COMBINATION FOR REDUCING ANTIMICROBIAL RESISTANCE USING A METHYLATION  
INHIBITOR IN COMBINATION WITH AN ANTIBIOTIC

### BACKGROUND OF THE INVENTION

#### Field of the Invention

10 The present invention relates generally to the  
fields of bacteria, antimicrobials and antibiotics. More  
particularly, it provides novel methods, kits, and  
combinations of antimicrobial agents and inhibitors, for  
15 use in reducing the resistance of bacteria and other  
microorganisms to antimicrobial agents. In particular,  
the invention provides for enhanced bacterial killing  
using a macrolide, lincosamide and streptogramin B (MLS)  
antibiotic in combination with an agent that inhibits  
20 methylation, as exemplified by inhibiting methylation or  
maturation of bacterial RNA.

#### Description of the Related Art

25 The first antibiotics were used clinically in the  
1940s and 1950s, and their use has been increasing  
significantly since this period. Although an invaluable  
advance, antibiotic and antimicrobial therapy suffers  
from several problems, particularly when strains of  
30 various bacteria appear that are resistant to  
antibiotics. Interestingly, bacteria resistant to  
streptomycin were isolated about a year after this  
antibiotic was introduced (Waksman, 1945).

35 The development of antibiotic resistance is a  
serious and life-threatening event of worldwide  
importance. For example, strains of *Staphylococcus* are  
known that are immune to all antibiotics except one  
(Travis, 1994). Such bacteria often cause fatal hospital

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infections. Among other drug resistant organisms are:  
pneumococci that cause pneumonia and meningitis;  
*Cryptosporidium* and *E. coli* that cause diarrhea; and  
enterococci that cause blood-stream, surgical wound, and  
5 urinary tract infections (Berkelman et. al., 1994).

Davies (1986) described seven basic biochemical  
mechanisms for naturally-occurring antibiotic resistance:  
(1) alteration (inactivation) of the antibiotic; (2)  
10 alteration of the target site; (3) blockage in the  
transport of the antibiotic; (4) by-pass of the  
antibiotic sensitive-step (replacement); (5) increase in  
the level of the inhibited enzyme (titration of drug);  
(6) sparing the antibiotic-sensitive step by endogenous  
15 or exogenous product; and (7) production of a metabolite  
that antagonizes action of inhibitor.

Certain bacteria become resistant to antibiotics by  
utilizing ribosomal mutations (Cunliffe, 1990), although  
20 some reports have stated that this type of resistance is  
of doubtful clinical significance (Spratt, 1994).  
Ribosomal mutations result in bacterial resistance to  
macrolide, lincosamide and streptogramin B (MLS)  
antibiotics, as has been observed in the resistance of  
25 various *Staphylococcus*, *Streptococcus*, *Enterococcus*,  
*Bacillus* and *Mycoplasma* strains to important antibiotics  
such as erythromycin (LeClercq & Courvalin, 1991).

The induction of erythromycin resistance generally  
30 leads to bacterial strains that express rRNA which does  
not bind to this type of antibiotic. Mainly,  
erythromycin resistance is due to the induction by  
macrolides of a methylase protein, which catalyzes the  
methylation of the binding site of erythromycin on the  
35 rRNA, thus preventing antibiotic binding. Despite  
intensive studies in this area, there remains few, if



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any, practical proposals as to how bacterial resistance to MLS antibiotics may be overcome.

#### SUMMARY OF THE INVENTION

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The present invention seeks to overcome these and other drawbacks inherent in the prior art by providing new combined compositions, medicaments, kits and uses thereof in reducing resistance to antimicrobials and antibiotics and for treating infections. The invention rests in the surprising use of one or more inhibitors of methylation in conjunction with an antimicrobial agent. The combination is used to impair the ability of a microorganism to methylate various substrates.

15

As the inventors contemplate that methylation, either at the DNA, RNA, protein or small molecule level, plays a role in all mechanisms of resistance of a microorganism to an antimicrobial agent, inhibition of methylation will be widely applicable to reducing resistance in any given microorganism or bacterium, and to treating a variety of diseases. For example, the organisms listed in Table 1 and Table 2 may be targeted using the antibiotics also listed in Table 1 and Table 2, amongst others, in combination with a methylation inhibitor. However, the invention is not limited to antibiotics and to bacteria alone, and is widely applicable to all microorganisms, including yeast and a variety of other pathogenic and even parasitic organisms.

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The invention therefore encompasses compositions, medicaments, kits and uses thereof which include a methylation inhibitor capable of inhibiting any process listed in Table 1, as exemplified by the processes of antibiotic alteration, target site alteration and antibiotic transport. Also, as methylation inhibitors act to alter gene expression and protein synthesis, the

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invention includes methylation inhibitors that prevent the by-pass of antibiotic sensitive-steps, prevent an increase in the amount of an inhibited enzyme, prevent the production of an alternative endogenous or exogenous product, and prevent the production of a metabolite that antagonizes the action of inhibitor.

One particular example of the applicability of the invention is in providing combinations and medicaments for use in reducing the resistance of bacteria to macrolide, lincosamide, and streptogramin B (MLS) antibiotics, or in enhancing the sensitivity of susceptible strains to such antibiotics. In this case, the inhibitor will primarily inhibit methylation of bacterial rRNA, thus rendering the bacterium susceptible to antibiotic rRNA binding and allowing bacterial protein synthesis to be inhibited.

The various embodiments of the invention include combined compositions, medicaments and kits for use in reducing the resistance of a microorganism to an antimicrobial agent, as exemplified by reducing the antibiotic resistance of an MLS-susceptible bacterium to an MLS antibiotic. Also provided are compositions, medicaments and therapeutic kits for killing microorganisms, such as bacteria, and particularly, MLS-susceptible bacteria; and their use for treating infections and diseases caused by microorganisms, including treating MLS-susceptible bacterial infections and diseases. By way of example only, certain infections that may be treated using the invention are listed in Table 3.

To reduce the resistance of a microorganism to an antimicrobial agent, as exemplified by reducing the resistance of a bacterium to an antibiotic, or to kill a microorganism or bacterium, one would generally contact

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the microorganism or bacterium with an effective amount of the antibiotic or antimicrobial agent in combination with an amount of a second agent effective to inhibit methylation in the microorganism or bacterium. In terms  
5 of killing or reducing the resistance of an MLS-susceptible bacterium, one would contact the bacterium with an effective amount of an MLS antibiotic in combination with an amount of a second agent effective to inhibit RNA methylation, synthesis and/or maturation  
10 in the bacterium. The terms "microorganism" and "bacterium" are used for simplicity and it will be understood that the invention is suitable for use against a population of microorganisms, i.e., "bacteria".

15 The microorganism, e.g., bacterium, or population thereof, may be contacted either *in vitro* or *in vivo*. Contacting *in vivo* may be achieved by administering to an animal (including a human patient) that has, or is suspected to have a microbial or bacterial infection, a  
20 therapeutically effective amount of pharmacologically acceptable antimicrobial agent formulation in combination with a therapeutic amount of a pharmacologically acceptable formulation of a second agent effective to inhibit methylation, e.g., effective to inhibit RNA  
25 methylation. The invention may thus be employed to treat both systemic and localized microbial and bacterial infections by introducing the combination of agents into the general circulation or by applying the combination, e.g., topically to a specific site, such as a wound or  
30 burn, or to the eye, ear or other site of infection.

An "effective amount of an antimicrobial agent or antibiotic" means an amount, or dose, within the range normally given or prescribed. Such ranges are well  
35 established in routine clinical practice and will thus be known to those of skill in the art. Appropriate oral and parenteral doses and treatment regimens are further

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detailed herein in Table 4 and Table 5. Supplementary information relating specifically to MLS antibiotics is also given in Example I and in Tables 6 and 7, which particularly concern erythromycin, lincomycin, clarithromycin and azithromycin. As this invention provides for enhanced microbial and/or bacterial killing, it will be appreciated that effective amounts of an antimicrobial agent or antibiotic may be used that are lower than the standard doses previously recommended when the antimicrobial or antibiotic is combined with a methylation inhibitor.

The "second agents" for use in the invention are generally methylation inhibitors, and are also referred to herein as "inhibitors" and "modifiers". The second agent inhibitors should be used in amounts effective to inhibit methylation in a microorganism or bacterium, as exemplified by an amount effective to inhibit RNA methylation, synthesis and/or maturation in an MLS-susceptible bacterium. Suitable amounts effective to inhibit methylation will be known, or readily identifiable, to those of skill in the art. Effective inhibitory amounts are the amounts that have previously been shown in the scientific literature to inhibit methylation generally or to inhibit a specific methylation step. In addition to the present disclosure and the references specifically incorporated herein, there is considerable scientific literature concerning methylation inhibitors that may be utilized in light of the inventors' discovery that such compounds may be effectively combined with antibiotics and other antimicrobial agents.

Amounts effective to inhibit methylation may also be measured, rather than identified from the published literature. Most simply, this is achieved by determining the amount effective to increase microbial or bacterial

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killing when used in combination with an antimicrobial agent, i.e., by determining an amount effective to reduce antimicrobial resistance. The determinations of effective inhibitory amounts and therapeutic doses will be routine to those of skill in the art given the teachings of the present disclosure, including the detailed methodology and the effective amounts of various agents disclosed, e.g., in Table 8 and throughout the detailed examples.

10

Naturally, in confirming the optimal therapeutic dose, first animal studies and then clinical trials would be conducted, as is routinely practiced in the art. Animal studies are common in the art and are further described herein (Example II) and in publications such as Lorian (1991, pp. 746-786, incorporated herein by reference) and Cleeland & Squires (incorporated herein by reference, from within the Lorian text).

15

The  $ID_{50}/IC_{50}$  ratio required for safe use of the proposed inhibitor-antimicrobial agent combinations will be assessed by determining the  $ID_{50}$  (median lethal toxic dosage) and the  $IC_{50}$  (median effective therapeutic dosage) in experimental animals. The optimal dose for human subjects is then defined by fine-tuning the range in clinical trials. In the case of  $ID_{50}$ , the inhibitor is usually administered to mice or rats (orally or intraperitoneal) at several doses (usually 4-5) in the lethal range. The dose in mg/kg is plotted against % mortality and the dose at 50% represents the  $ID_{50}$  (Klaassen, 1990). The  $IC_{50}$  is determined in a similar fashion as described by Cleeland & Squires (1991).

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In a clinical trial, the therapeutic dose would be determined by maximizing the benefit to the patient, whilst minimizing any side-effects or associated toxicities. Throughout the detailed examples, various

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therapeutic ranges are listed. Unless otherwise stated, these ranges refer to the amount of an agent to be administered orally.

5           In optimizing a therapeutic dose within the ranges disclosed herein, one would not use the upper limit of the range as the starting point in a clinical trial due to patient heterogeneity. Starting with a lower or mid-range dose level, and then increasing the dose will limit  
10 the possibility of eliciting a toxic or untoward reaction in any given patient or subset of patients. The presence of some side-effects or certain toxic reactions per se would not, of course, limit the utility of the invention, as it is well known that most beneficial drugs also  
15 produce a limited amount of undesirable effects in certain patients. Also, a variety of means are available to the skilled practitioner to counteract certain side-effects, such as using vitamin B<sub>12</sub> in association with N<sub>2</sub>O treatment (Ostreicher, 1994).

20

It is important to note that the invention does not require the identification of novel methylation inhibitors, but rather concerns the new and surprisingly  
- effective use of various compounds, already known to have  
25 certain functional properties, in combination with antimicrobial agents or antibiotics. Neither does the invention require the identification of a precise molecular target, or of all targets, that a given methylation inhibitor may act upon.

30

It is not necessary therefore to determine methylation levels in a microorganism or bacterium to practice the invention, or to identify all the enzymes or other target molecules that are altered by the action of  
35 the inhibitor. All that is required is to use an antimicrobial agent in combination with an agent known to

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be, or suspected to be, capable of inhibiting methylation and to determine whether increased killing results.

5 Zak & Sande (1981) reported on the correlation  
between the *in vitro* and *in vivo* activity of a 1000  
compounds that were randomly screened for antimicrobial  
activity. The important finding in this study is that  
negative *in vitro* data is particularly accurate, with the  
negative *in vitro* results showing more than a 99%  
10 correlation with negative *in vivo* activity. This is  
meaningful in the context of the present invention as one  
or more *in vitro* assays will be conducted prior to using  
any given combination in a clinical setting. Any  
negative result obtained in such an assay will thus be of  
15 value, allowing efforts to be more usefully directed.

Although the invention was developed, in part, from  
a consideration of various biochemical interactions and  
pathways, an understanding of the precise mechanism by  
20 which any given compound functions to reduce methylation  
in a microorganism, as measured by enhanced killing, is  
not relevant to practicing the invention. Therefore,  
effective methylation inhibitors, include those compounds  
that inhibit methylation both directly and indirectly.  
25 Many different enzymes and biochemical steps may be  
inhibited, or otherwise altered to the detriment of the  
microorganism or bacterium, as will be clear from the  
detailed examples.

30 This invention is considered to be particularly  
suitable for use in increasing the effectiveness of MLS  
antibiotics against, e.g., gram-positive staphylococcus,  
streptococcus, enterococcus and bacillus, gram-negative  
cocci and gram-negative aerobes.

35

Bacteria that may thus be attacked include those  
listed in Tables 6 and 7, and also, *Staphylococcus* spp.,

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*S. sanguis*, *Corynebacterium diphtheria*, *Bacteroides* spp.,  
*B. ovatus*, *Clostridium* spp., *C. difficile*, *B. subtilis*,  
*Lactobacillus* spp., *Campylobacter* spp., *Propionibacterium*  
spp., *Mycoplasma* spp., *Fusobacterium*, *Corynebacterium*,  
5 *Veillonella*, *S. fecalis*, *Nocardia farcinica*,  
*Actinobacillus actinomycetemcomitans*, Group A and B  
streptococci, *Bacillus stearothermophilus*, or *Pseudomonas*  
*aeruginosa*.

10 The second agent inhibitors may thus be effectively  
combined with one or more macrolide antibiotics, such as  
erythromycin, azithromycin, clarithromycin,  
roxithromycin, oleandomycin, spiramycin, josamycin,  
miocamycin, midecamycin, rosaramycin, troleandomycin,  
15 flurithromycin, rokitamycin or dirithromycin; one or more  
lincosamide antibiotics, such as lincomycin, clindamycin,  
celesticetin; or one or more streptogramin B antibiotics,  
such as pristinamycin or virginiamycin. Erythromycin,  
azithromycin, clarithromycin, lincomycin and clindamycin  
20 are currently the most preferred.

Suitable inhibitory second agents include those that  
inhibit RNA methyltransferase enzymes, as described in  
- Example IV. These agents are exemplified by  
25 S-adenosylhomocysteine (SAH) and analogues; homocysteine;  
adenine derivatives, such as adenine phosphate, adenine  
sulfate, adenine-N<sup>1</sup>-oxide, 6-methyladenine,  
6-mercaptopadenine, with dimethylallyladenine and  
isopentenyladenosine being particularly preferred; and  
30 SAM analogues and derivatives, such as methyl, ethyl,  
n-propyl, allyl, n-butyl, n-pentyl, n-octyl and  
6-amino-1-hexyl nitrogen analogues of SAM, and  
S-inosyl-L-methionine, S-adenosyl-(5')-3-methylthio-  
propylamine and 5'-methylthioadenosine.

35

Further inhibitors of RNA methyltransferases include  
sinefungin and analogues and metabolites, such as A9145C,



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5'-S-methylthioadenosine and cyclosinefungin;  
nicotinamide and analogues, such as methylnicotinamide;  
and polyinosinate. Specific examples of suitable  
compounds include S-N<sup>6</sup>-methyladenosylhomocysteine;  
5 S-aristeromycinyl-L-homocysteine and analogues thereof,  
such as 3-deazaaristeromycinyl-D-homocysteine;  
5'-deoxy-5'-S-isobutyl-adenosine (SIBA) and ISOSIBA;  
S-tubercidinylhomocysteine (STH) and analogues; MTA and  
xylosyladenine.

10

It will be recognized that many compounds will exert  
multiple effects on a microorganism or bacterial cell.  
For example, SAH analogues and tubercidin act at various  
points in methylation pathways. The division of the  
15 useful agents into certain groups is thus not intended to  
be an exact and non-overlapping scientific division.  
Rather such information is set forth to assist those in  
the art with understanding certain scientific data that  
were considered by the inventors.

20

Second agents that inhibit S-adenosylhomocysteine  
(SAH) hydrolase (SAHH) are also useful, as described in  
Example V, and exemplified by adenosine and analogues.  
Useful adenosine analogues include the first generation  
25 agents 9(S)-(2,3-dihydroxypropyl)adenine [(S)-DHPA], D-  
eritadine, (R,S)-3-adenine-9-yl-2-hydroxypropanoic acid  
[(R,S)-AHPA], adenosine (Ado) dialdehyde, 3-  
deazaadenosine (c<sup>3</sup>-Ado), aristeromycin (Ari) and  
neplanocin A (NPA or NpcA).

30

Second generation analogues are more preferred and  
include dihydroxycyclopentenyladenine (DHCEA),  
dihydroxycyclopentenyl-3-deazaadenine (c<sup>3</sup>-DHCEA),  
dihydroxycyclopentanyladenine (DHCAa),  
35 dihydroxycyclopentanyl-3-deazaadenine (c<sup>3</sup>-DHCAa),  
3-deazaneplanocin A (c<sup>3</sup>-NpcA), 3-deazaaristeromycin (c<sup>3</sup>-  
Ari), carbocyclic-3-deazaadenosine (C-c<sup>3</sup>Ado), 6'-

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C-methylneplanocin A, 2'-deoxyadenosine and tubercidin. Other useful analogues in this category include ribavirin, pyraazofurin, 2'-deoxy-2'-chloroadenosine, isopentenyladenosine and methylthioadenosine (MTA).

5

Other useful SAH hydrolase inhibitors include 9- $\beta$ -arabinofuranosyladenine (Ara-A, vidarabine) and 2'-Deoxyadenosine, of which Ara-A is already approved by the FDA. Aristeromycin analogues, including

10 aristeromycin itself and N-methylaristeromycin, 8-azaaristeromycin and 3-deazaaristeromycin, and their dialdehyde and diol derivatives are also effective inhibitors, as are ( $\pm$ )-5-Noraristeromycin and its 2,6-diamino-analogue. Further useful aristeromycinyl

15 analogues with reduced toxicity include 2'-deoxy-, 3'-deoxy-, 3'-amino-3'-deoxy-, 3'-amino-3'-deoxyarabinofuranosyl, 6'-hydroxy, 6'-mercapto, 8'-bromo, 8-hydroxyaristeromycin, aristeromycin-3'-cyclic phosphate and aristeromycin-6'-cyclic phosphate, all of

20 which may be used in the present invention.

Certain structural analogues of SAH with modification in the amino acid, base or sugar portion of the molecule may be used to inhibit SAH hydrolase.

25 Useful analogues of this class include 2-fluoro-S-adenosylhomocysteine (2-FSAH), S-Adenosyl-L-homocysteine sulfoxide, S-Adenosyl-L-homocysteine sulfone, S-aristeromycinyl-L-homocysteine, 5'-S-(3-carboxyl-4-nitrophenyl)thioadenosine and 5'-S-(methyl)-

30 5'-S-(butyl)thioadenosine.

S-adenosylmethionine (SAM) synthetase (SAM) is another target that may be inhibited, e.g., by using second agents such as cycloleucine (1-aminocyclopentane-1-carboxylic acid) and analogues; mono, bi- and

35 tri-cyclic amino acid structural and conformational analogues of L-methionine; and ATP/methionine analogues,

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such as AMPNPP. Particular examples of cycloleucine and methionine analogues include 1-aminocyclobutanecarboxylic acid, 1-aminocyclohexanecarboxylic acid, L-2-amino-4-hexanoic acid, (Z)-L-2-amino-5-chloro-trans-4-hexanoic acid, L-ethionine, seleno-DL-ethionine, the (1R,2R,4S) isomer of 2-aminonorbornane-2-carboxylic acid, the (1R,2R,4S) isomer of 2-amino-5,6-exo-trimethylenenorbornane-2-carboxylic acid, 3-aminobicyclo[3.2.0]heptane-3-carboxylic acid and, preferably, (+)-2-aminobicyclo[2.1.1]hexane-2-carboxylic acid.

Further suitable inhibitors of (SAM) synthetase include L-2-amino-4-methoxy-cis-but-3-enoic acid (L-cis-AMB), L-2-amino-4-methylthio-cis-but-3-enoic acid (L-cisAMTB) and covalent methionine-ATP adducts such as 5' (R) - (C) - [(L-homocysteine-S-yl) methyl]adenosine 5' - ( $\beta$ ,  $\gamma$ ) - imidotriphosphate.

Agents that inhibit, by any mechanism, the synthesis of glutathione form another group of SAM inhibitors. Inhibitors of glutathione synthetase are examples of this class, and include buthionine sulfoximine, 7,8-dihydrofolate,  $\alpha$ -aminomethylglutarate, SAPH-3 and diethyl maleate. Methotrexate and trimethoprim, that inhibit DHFR, also act to reduce glutathione synthetase activity.

Other agents that may be employed are methionine synthetase (MS) and homocysteine transmethylase inhibitors, such as nitrous oxide ( $N_2O$ ), as commonly employed in dentistry. Adenosine deaminase (ADA), and even adenosine kinase, inhibitors may also be used. ADA inhibitors include, e.g., coformycin, deoxycoformycin (pentostatin) and isomers, 1,6-dihydro-6-hydroxymethylpurine nucleoside, erythro-9-(2-hydroxy-3-nonyl)adenine, 6-methylaminopurine

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riboside and 2'-3'-isopropylidene-adenosine. Compounds that inhibit the enzyme after ADA, purine nucleoside phosphorylase, are also contemplated for use as they will lead to a build up of inosine.

5

Dihydrofolate reductase (DHFR) inhibitors, such as the clinically used drugs methotrexate (MTX), aminopterin and trimethoprim, and 4,6-diamino-2,2-dimethyl-s-triazine and 2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine analogues (e.g., NSC 127755 and 139105) may be used, as may inhibitors of dihydropteroate synthetase, such as sulfanilamide, sulfadiazine, sulfamethoxazole, sulfisoxazole and sulfacetamide that also inhibit DHFR.

15

<sup>5</sup>N-Methyltetrahydrofolate (5-CH<sub>3</sub>-FH<sub>4</sub>) is used as a methyl donor in the last step of methionine synthesis from homocysteine in many bacterial reactions.

20

Inhibition of 5-CH<sub>3</sub>-FH<sub>4</sub> synthesis will thus cause a reduction in SAM synthesis. The inventors therefore contemplate that agents that inhibit the production of any of the biosynthetic precursors of 5-CH<sub>3</sub>-FH<sub>4</sub>, or those that otherwise exert a negative influence on any of the 5-CH<sub>3</sub>-FH<sub>4</sub> biosynthetic steps, will also be useful in the present invention. Exemplary inhibitors are those listed above such as MTX, NSC 127755, sulfonamides, and the like.

25

Compounds that inhibit polyamine synthesis are also useful. These include compounds such as α-methylornithine, 1,3-diaminopropan-2-ol, difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA). Also included are those compounds that inhibit specific enzyme targets, such as ornithine decarboxylase (ODC), arginine decarboxylase (ADC) and SAM Decarboxylase (SAM-DC). Inhibitors of SAM-DC include AMA, MHZPA, MAOEA, AbeAdo (MDL 73811), AdoMac, MGBG, CGP-39'937 and CGP-33'829, as shown in Example X; inhibitors of ODC

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include eflornithine and other compounds described by McCann & Pegg (1992) and Pegg & McCann (1992); and inhibitors arginine decarboxylase include, by way of example, DL- $\alpha$ -(difluoromethyl)arginine.

5

Compounds such as 5-azacitidine, cordycepin (3'-deoxyadenosine), toyocamycin and analogues, that inhibit RNA maturation, are also contemplated for use. Antisense oligonucleotides may also be employed to inhibit the induction of bacterial methylase synthetase or as decoys that prevent the methylation of rRNA. Antisense oligonucleotides may be advantageously delivered using liposomes.

10

In the preparation of medicaments for the treatment of animals or human patients, there are various appropriate formulations and treatment regimens that may be used. For example, the antimicrobial compound(s) and second agent(s) may be administered to an animal simultaneously, e.g., in the form of a single composition that includes the microbial/antibiotic and second agent, or by using at least two distinct compositions. The antimicrobial agent could also be administered to the animal prior to the second agent, although it is currently more preferred to give the second agent inhibitor prior to the antimicrobial agent.

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The invention also provides novel compositions and medicaments that contain a combination of antimicrobials and second agents, not previously proposed for combined use, dispersed in a pharmacologically acceptable formulation. The antimicrobial agents and inhibitory second agents may be formulated and administered in any pharmacologically acceptable vehicle, such as parenteral, topical, liposomal, nasal or ophthalmic preparations, with formulations designed for oral administration being currently preferred due to their ease of use.

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Multiple combinations may also be used, such as more than one antimicrobial agent used with one second agent or more than one second agent used with a given antimicrobial. Different classes of antimicrobial agents and second agents may be combined, naturally following the general guidelines known in the art regarding drug interactions. Preferred combinations will be those in which inhibitors from different classes are used. Typically, between one and about five distinct antimicrobial agents are contemplated for use along with between one and about ten distinct second agents. Combinations including at least one MLS antibiotic are currently preferred as there appears to be a particular need in the art for an improvement in MLS antibiotic therapy.

Currently, one preferred group of inhibitory agents includes DHPA, D-eritadine, (R,S)-AHPA, adenosine dialdehyde,  $c^3$ -Ado, aristeromycin and neplanocin A; with DHCEA,  $c^3$ -DHCEA, DHCA,  $c^3$ -DHCA,  $c^3$ -NpCA,  $c^3$ -Ari, C- $c^3$ Ado, 6'-C-methylneplanocin A, tubercidin and Ara-A being more preferred. Further preferred compounds are polyinosinate, L-cis-AMB, L-cis-AMTB, buthionine sulfoximine, methotrexate, trimethoprim,  $N_2O$ , NSC 127755, NSC 139105, DFMO, DFMA, coformycin, deoxycoformycin and sulfonamides.

Further embodiments of the invention include therapeutic kits that comprise, in suitable container means, a pharmaceutical formulation of at least one antimicrobial agent and a pharmaceutical formulation of at least one microbial methylation inhibitor. The antibiotics and inhibitory second agents may be contained within a single container means, or a plurality of distinct containers may be employed.

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BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

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FIG. 1. Erythromycin susceptibility for *Streptococcus aureus* 6538 (erythromycin susceptible) where ethionine, cycloleucine and trimethoprim are pre-mixed with brain heart infusion (BHIA, filled bars) or trypticase soya agar (TSA, unfilled bars).

15

FIG. 2. Erythromycin susceptibility for *S. aureus* 27660 (erythromycin resistant), where ethionine (0.051M), cycloleucine (0.10M) and trimethoprim (0.26mM) are pre-mixed with BHIA (filled bars) or TSA (unfilled bars).

20

FIG. 3. Erythromycin disc-susceptibility for *S. aureus* 27660 (erythromycin resistant), pre-incubated with inhibitor-containing discs. ((-----) denotes BHIA(E) and (\_\_\_\_\_) denotes BHIA(E)<sub>c</sub>)

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Antimicrobial combinations are well known and are most frequently used to provide broad-spectrum empirical coverage in the treatment of patients who are seriously ill and who may be septicemic. Combinations are also chosen because an identified pathogen is resistant to inhibition and/or killing by conventional doses of a single antibiotic, but in contrast is susceptible to the combination (Eliopoulos & Moellering, 1991).

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Lorian (1991) indicated that there are generally four accepted mechanisms of antibacterial synergism, namely, (1) serial or sequential inhibition of a common biochemical pathway (e.g. trimethoprim-sulfamethoxazole);  
5 (2) inhibition of protective bacterial enzyme (clavulanic acid plus a B-lactamase-susceptible penicillin); (3) combination of cell wall-active agents (e.g. ampicillin and ampicillin); and (4) use of cell wall-active agents to enhance the uptake of other antimicrobials (e.g.  
10 penicillin and streptomycin). The present invention provides a novel synergistic option for antimicrobial treatment.

#### Antibiotic Resistance

15

Seven basic biochemical mechanisms for naturally-occurring antibiotic resistance have been described (Davies, 1986), namely alteration of the antibiotic; alteration of the target site; block in the transport of  
20 the antibiotic; by-pass of the antibiotic sensitive-step; increasing the level of the inhibited enzyme; the cell is spared the antibiotic-sensitive step by endogenous or exogenous product; and the production of a metabolite that antagonizes action of inhibitor. The same general  
25 concepts also apply to microorganisms other than bacteria.

Antibiotic and antimicrobial resistance can be spread vertically and horizontally by plasmids and  
30 transposons. The inventors propose that methylation reactions play an active role in both plasmids and transposons, and that methylation inhibitors will modulate general antibiotic resistance. Butler & Gotschlich (1991) indicated that high-frequency  
35 mobilization of broad-host-range plasmids into *Neisseria gonorrhoea* requires methylation in the donor. Additionally, they concluded that methylation/restriction



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systems was a major barrier against deficient conjugal transfer between *N. gonorrhoea* and heterologous hosts.

5 This invention therefore encompasses methods to reduce antimicrobial resistance, caused by any of the seven mechanisms described above, using a combination of the antimicrobial agent and one or more inhibitor(s) that modify or influence methylation and the associated processes. A list of antibiotics that have developed  
10 antibiotic resistance by one or more of these mechanisms is listed in Table 1 (taken from Lorian, 1991), all of which are intended to be combined with a methylation inhibitor, as described herein, thereby achieving enhanced microbial killing.

15 Table 2 (taken from Reese & Betts, 1993; Med. Let., 1992), lists the antibiotics generally preferred for use against a given pathogenic bacterium. It is contemplated that the effectiveness of all the antibiotics listed in  
20 Table 2 will be increased upon combination with a methylation inhibitor. Table 3 (also taken from Reese & Betts, 1993), itemizes the common pathogenic bacteria that are implicated in focal infections. The present invention is thus contemplated for use against all such  
25 infections.

TABLE 1  
MECHANISMS OF RESISTANCE TO ANTIMICROBIAL AGENTS

Antimicrobial Agent	Mechanisms Causing Resistance	Examples of Organisms
<i>Aminoglycosides</i>	Modifying enzymes: acetyltransferases, adenylyl- transferases (nucleotidyl- transferases), phosphotransferases Ribosomal resistance (streptomycin, spectinomycin)	Enterobacteriaceae, <i>P.</i> <i>aeruginosa</i> , <i>S. aureus</i> , <i>E.</i> <i>faecalis</i>
	Inadequate drug transport	<i>E. faecalis</i> , Enterobacteriaceae, <i>M.</i> <i>tuberculosis</i> , <i>P.</i> <i>aeruginosa</i> <i>E. faecalis</i> , <i>P.</i> <i>aeruginosa</i> , anaerobes
$\beta$ -Lactams	Enzymatic inactivation	<i>S. aureus</i> , <i>E. faecalis</i> , Enterobacteriaceae, <i>P.</i> <i>aeruginosa</i> , <i>Neisseria</i> spp., <i>H. influenzae</i> <i>S. pneumoniae</i> , <i>N.</i> <i>gonorrhoeae</i> , <i>S. aureus</i> , <i>P. aeruginosa</i>
	Low affinity PBPs	<i>P. aeruginosa</i>
	Lack of penetration through outer membrane	<i>P. aeruginosa</i> , Enterobacteriaceae

TABLE 1 (continued)

Antimicrobial Agent	Mechanisms Causing Resistance	Examples of Organisms
Chloramphenicol	Acetylation	Enterobacteriaceae, <i>S. aureus</i> , streptococci, <i>Bacteroides uniformis</i> , <i>P. aeruginosa</i>
Clindamycin, erythromycin, lincomycin	Lack of penetration	
	Ribosomal resistance due to methylation of rRNA Inactivation by esterase Decreased penetration	Streptococci, <i>E. faecalis</i> , Enterobacteriaceae Enterobacteriaceae <i>S. hominis</i>
Fluoroquinolones	Decreased uptake	Enterobacteriaceae, <i>P. aeruginosa</i> , staphylococci
	Altered target site (DNA gyrase)	Enterobacteriaceae, <i>P. aeruginosa</i>
Lincomycin	Inactivation	<i>S. aureus</i>
Sulfonamides	Synthesis of an altered or alternative target site (dihydropteroate synthetase) Lack of penetration Overproduction of PABA	Enterobacteriaceae, <i>Neisseria</i> spp., <i>P. aeruginosa</i> Anaerobes <i>Neisseria</i> , <i>S. aureus</i>

TABLE 1 (continued)

Antimicrobial Agent	Mechanisms Causing Resistance	Examples of Organisms
Tetracycline	Drug efflux	Enterobacteriaceae, staphylococci, streptococci
	Protection of ribosome from tetracycline	Streptococci, <i>E. faecalis</i> , <i>Neisseria</i> spp., <i>Mycoplasma</i> spp.
	Inactivation	Cryptic gene found in <i>B. fragilis</i> , expressed resistance in <i>E. coli</i>
Trimethoprim	Synthesis of an altered or alternative target site (dihydrofolate reductase) Lack of penetration Ability to use alternative pathway Overproduction of dihydrofolate reductase	Enterobacteriaceae, <i>V. cholerae</i> , staphylococci  <i>P. aeruginosa</i> Enterococci <i>H. influenzae</i>
Vancomycin	?	Pediococci, <i>Leuconostoc</i> spp. (intrinsic)
	?Blocking of target site	Enterococci (acquired)

**TABLE 2**  
**ANTIBIOTICS OF CHOICE FOR COMMON PATHOGENS**

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<b>Gram-positive cocci</b>		
<i>Staphylococcus aureus</i> or <i>S. epidermidis</i> Non-penicillinase- producing	Penicillin	A first-generation cephalosporin, vancomycin, imipenem, or clindamycin; a fluoroquinolone <sup>b</sup>
Penicillinase- producing	Penicillinase-resistant penicillin (e.g., oxacillin or nafcillin)	A first-generation cephalosporin, vancomycin, clindamycin, imipenem, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, ampicillin-sulbactam; a fluoroquinolone <sup>b</sup>
Methicillin-resistant	Vancomycin with or without gentamicin and/or rifampin	TMP-SMZ, minocycline

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
Streptococci Group A, C, G	Penicillin	A cephalosporin <sup>a</sup> , vancomycin, erythromycin; clarithromycin; azithromycin; clindamycin
Group B	Penicillin (or ampicillin)	A cephalosporin <sup>a</sup> , vancomycin, or erythromycin
Enterococcus Endocarditis or other serious infection	Penicillin (or ampicillin) with gentamicin	Vancomycin with gentamicin
Uncomplicated urinary tract infection	Ampicillin or amoxicillin	A fluoroquinolone, nitrofurantoin
Viridans group	Penicillin G (with or without gentamicin)	A cephalosporin <sup>a</sup> , vancomycin
<i>S. bovis</i>	Penicillin G	A cephalosporin <sup>a</sup> , vancomycin
<i>S. pneumoniae</i>	Penicillin G	A cephalosporin <sup>a</sup> , erythromycin, chloramphenicol, vancomycin

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<b>Gram-negative cocci</b>		
<i>Neisseria gonorrhoeae</i>	Ceftriaxone	Spectinomycin, a fluoroquinolone, cefoxitin, cefixime, cefotaxime (see Appendix E)
<i>N. meningitidis</i>	Penicillin G	Third-generation cephalosporin, chloramphenicol
<i>Moraxella</i> ( <i>Branhamella</i> ) <i>catarrhalis</i>	TMP-SMZ	Amoxicillin-clavulanic acid; an erythromycin; clarithromycin azithromycin, cefuroxime, cefixime, third-generation cephalosporin, tetracycline
<b>Gram-positive bacilli</b>		
<i>Clostridium perfringens</i> (and <i>Clostridium</i> sp.)	Penicillin G	Chloramphenicol, metronidazole, or clindamycin
<i>Listeria monocytogenes</i>	Ampicillin with or without gentamicin	TMP-SMZ
<b>Gram-negative bacilli</b>		

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<i>Acinetobacter</i>	Imipenem	Tobramycin, gentamicin, or amikacin, usually with ticarcillin or piperacillin (or similar agent); TMP-SMZ
<i>Aeromonas hydrophila</i>	TMP-SMZ	Gentamicin, tobramycin; imipenem; a fluoroquinolone
<i>Bacteroides</i> <i>Bacteroides</i> sp. (oropharyngeal)	Penicillin G	Clindamycin, ceftioxin, metronidazole, chloramphenicol, cefotetan, ampicillin-sulbactam
<i>B. fragilis</i> strains (gastrointestinal strains)	Metronidazole	Clindamycin; ampicillin-sulbactam; imipenem; ceftioxin <sup>c</sup> ; cefotetan <sup>c</sup> ; ticarcillin-clavulanic acid; piperacillin <sup>c</sup> ; chloramphenicol; cefmetazole <sup>c</sup>
<i>Campylobacter fetus</i> , <i>jejuni</i>	A fluoroquinolone (adults) or an erythromycin	A tetracycline, gentamicin



TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<i>Enterobacter</i> sp.	Imipenem	An aminoglycoside and piperacillin or ticarcillin or mezlocillin; a third-generation cephalosporin <sup>d</sup> ; TMP-SMZ; aztreonam; a fluoroquinolone
<i>Escherichia coli</i> Uncomplicated urinary tract infection Recurrent or systemic infection	TMP-SMZ  A cephalosporin <sup>e</sup>	A cephalosporin or a fluoroquinolone  Ampicillin with or without an aminoglycoside, TMP-SMZ, oral fluoroquinolones useful in recurrent infections, ampicillin-sulbactam, ticarcillin-clavulanic acid, aztreonam

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<i>Haemophilus influenzae</i> (coccobacillary) Life-threatening infections Upper respiratory infections and bronchitis	Cefotaxime or ceftriaxone  TMP-SMZ	Chloramphenicol; cefuroxime for pneumonia) Ampicillin or amoxicillin; cefuroxime; a sulfonamide with or without an erythromycin; cefuroxime-axetil; third-generation cephalosporin, amoxicillin- clavulanic acid, cefaclor, tetracycline; clarithromycin; azithromycin
<i>Klebsiella pneumoniae</i>	A cephalosporin <sup>e</sup>	An aminoglycoside, imipenem, TMP- SMZ, ticarcillin-clavulanic acid, ampicillin-sulbactam, aztreonam, a fluoroquinolone; amoxicillin- clavulanic acid
<i>Legionella</i> spp.	Erythromycin with rifampin	TMP-SMZ; clarithromycin; azithromycin; ciprofloxacin

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<i>Pasteurella multocida</i>	Penicillin G	Tetracycline, cefuroxime, amoxicillin-clavulanic acid, ampicillin-sulbactam
<i>Proteus</i> sp.	Cefotaxime, ceftizoxime, or ceftriaxone	An aminoglycoside; ticarcillin or piperacillin or mezlocillin; TMP-SMZ; amoxicillin-clavulanic acid; ticarcillin-clavulanic acid, ampicillin-sulbactam; a fluoroquinolone; aztreonam; imipenem
<i>Providencia stuartii</i>	Cefotaxime, ceftizoxime, or ceftriaxone	Imipenem; an aminoglycoside often combined with ticarcillin or piperacillin or similar agent; ticarcillin-clavulanic acid; TMP-SMZ, a fluoroquinolone; aztreonam

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<i>Pseudomonas aeruginosa</i> (nonurinary tract infection)	Gentamicin or tobramycin or amikacin (combined with ticarcillin, piperacillin, etc. for serious infections) Ciprofloxacin	An aminoglycoside and ceftazidime; imipenem, or aztreonam plus an aminoglycoside; ciprofloxacin
(urinary tract infections)		Carbenicillin; ticarcillin, piperacillin, or mezlocillin; ceftazidime; imipenem; aztreonam; an aminoglycoside
<i>Pseudomonas cepacia</i>	TMP-SMZ	Ceftazidime, chloramphenicol
<i>Salmonella typhi</i>	Ceftriaxone	Ampicillin, amoxicillin, TMP-SMZ, chloramphenicol; a fluoroquinolone
Other species	Cefotaxime or ceftriaxone	Ampicillin or amoxicillin, TMP-SMZ, chloramphenicol; a fluoroquinolone
<i>Serratia</i>	Cefotaxime, ceftizoxime, or ceftriaxone <sup>f</sup>	Gentamicin or amikacin; imipenem; TMP-SMZ; ticarcillin, piperacillin, or mezlocillin; aztreonam; a fluoroquinolone
<i>Shigella</i>	A fluoroquinolone	TMP-SMZ; ceftriaxone; ampicillin

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice <sup>a</sup>	Alternative Agents <sup>a</sup>
<i>Vibrio cholerae</i> (chlorea)	A tetracycline	TMP-SMZ; a fluoroquinolone
<i>Vibrio vulnificus</i>	A tetracycline	Cefotaxime
<i>Xanthomonas</i> ( <i>Pseudomonas</i> ) <i>maltophilia</i>	TMP-SMZ	Minocycline, ceftazidime, a fluoroquinolone
<i>Yersinia enterocolitica</i>	TMP-SMZ	A fluoroquinolone; an aminoglycoside; cefotaxime or ceftizoxime
<i>Yersinia pestis</i> (plague)	Streptomycin	A tetracycline; chloramphenicol; gentamicin

Key: TMP-SMZ - trimethoprim-sulfamethoxazole.

<sup>a</sup> Choice presumes susceptibility studies indicate that the pathogen is susceptible to the agent.

<sup>b</sup> The experience with fluoroquinolone use in staphylococcal infections is relatively limited.

The fluoroquinolones should be used only in adults.

<sup>c</sup> Up to 15-20% of strains may be resistant.

<sup>d</sup> *Enterobacter* spp. may develop resistance to the cephalosporins.

<sup>e</sup> Specific choice will depend on susceptibility studies. Third-generation cephalosporins may be exquisitely active against many gram-negative bacilli (e.g., *E. coli*, *Klebsiella* sp.). In some geographic areas, 20-25% of community-acquired *E. coli* infections may be resistant to ampicillin (amoxicillin).

<sup>f</sup> In severely ill patients, this is often combined with an aminoglycoside while awaiting susceptibility data.

TABLE 3  
COMMON PATHOGENS IN FOCAL INFECTIONS

Presumed location of infection	Common pathogens	Gram stain characteristics of exudate-if available
Urinary tract infections	Community-acquired: <i>Escherichia coli</i> Recurrent or nosocomial: <i>E. coli</i> , <i>Klebsiella</i> , <i>Proteus</i> , <i>Pseudomonas</i> sp. Enterococci	GNB GNB GPC
Intravenous catheter phlebitis and/or sepsis Peripheral catheter	<i>Staphylococcus aureus</i> or <i>S.</i> <i>epidermidis</i> <i>Klebsiella</i> , <i>Enterobacter</i> , <i>Pseudomonas</i> sp. <i>Candida</i> sp., <i>S. aureus</i> , <i>S.</i> <i>epidermidis</i> , enterococci <i>Klebsiella</i> , <i>Enterobacter</i> sp., etc.	GPC GNB Budding yeast; GPC GNB
Hyperalimentation line		
Arteriovenous shunt	<i>S. aureus</i> , <i>S. epidermidis</i>	GPC
Septic bursitis	<i>S. aureus</i>	GPC

TABLE 3 (continued)

Presumed location of infection	Common pathogens	Gram stain characteristics of exudate-if available
Biliary tract	<i>E. coli</i> , <i>Klebsiella</i> sp., and enterococci; <i>Bacteroides fragilis</i> (in elderly patients), <i>Clostridia</i> sp.	
Intra-abdominal abscess, peritonitis, or large bowel perforation; diverticulitis <sup>a</sup>	<i>E. coli</i> <i>B. fragilis</i>  <i>Klebsiella</i> sp. (Enterococci)	GNB GNB (thin, irregularly stained) GNB GPC
Burn wounds	Early: <i>S. aureus</i> , streptococci Later: gram-negative bacilli, fungi	
Cellulitis, wound and soft tissue infections	<i>S. aureus</i> Streptococci <i>Clostridium</i> sp.	GPC GPC GPB
Meningitis	See Appendix C	
Pneumonia	See Appendix D	

TABLE 3 (continued)

Presumed location of infection	Common pathogens	Gram stain characteristics of exudate if available
Pelvic abscess, postabortal or postpartal	Anaerobic streptococci <i>B. fragilis</i> <i>Clostridium</i> sp. <i>E. coli</i> Enterococci	GPC GNB (thin, irregularly stained) GPB GNB GPC
Septic arthritis	<i>S. aureus</i> <i>Haemophilus influenzae</i> (in children younger than 6 yr) Group B streptococci (in neonates) Gram-negative organisms <sup>b</sup>	GPC GNC GPC GNB
Acute osteomyelitis	<i>S. aureus</i> <i>H. influenzae</i> (in children younger than 6 yr) Group B streptococci (in neonates) Gram-negative organisms <sup>b</sup>	GPC GNC GPC GNB

Key: GNB = gram negative bacilli; GPC = gram-positive cocci; GPB = gram-positive bacilli; GNC = gram-negative coccobacilli.

<sup>a</sup>The precise role of enterococci in intra-abdominal infections is unclear. In mild to moderate infections, it may not be necessary to provide antibiotic activity against enterococci.

<sup>b</sup>In high-risk patients (e.g., immunocompromised, elderly, IV drug abusers, diabetics, debilitated patients).



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As a particular example, the  $\beta$ -lactams will be described. To protect against the constantly changing hostile environment, certain bacteria have developed a cell membrane which includes complex carbohydrate-peptide structures, assembled from individual carbohydrate and amino acid monomers in the cytoplasm and then transported out across the growing membrane for assembly. After export and attachment to the growing cell membrane, a free C-terminal end of a peptide is cross-linked to other chains to give the required rigidity to the cell membrane. Many  $\beta$ -lactam antibiotics block this step which occurs on the outside of the membrane, and which leads to weakening of the cell membrane and eventual cell-lysis (Rapley & Walker, 1993).

To compensate, the bacterium produces lactamase enzymes that hydrolyse an amide bond in the  $\beta$ -lactam antibiotics to inactivate them. In order to achieve this resistance, lactamase synthesis is induced in the cytoplasm and this protein has to be exported to the outer face of the membrane to be effective since the site of  $\beta$ -lactamase antibiotics are on the outside of the membrane. In gram positive bacteria, the  $\beta$ -lactamases are targeted first to the periplasmic space by a "peptide" leader sequence (Rapley & Walker, 1993).

As will now be indicated, many of the processes described in this resistance mechanism are controlled by methylation. In assessing this collection of diverse information, the present inventors developed the idea that antimicrobial resistance could be reduced in practice using methylation inhibitors. Although many scientific articles have analyzed the mechanisms underlying antibiotic resistance, the idea of using methylation inhibitors has not been proposed previously.

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The inventors now suggest that for the seven known antibiotic resistant mechanisms to operate, one prerequisite generally is the induction of the synthesis of at least one protein. They therefore predict that  
5 methylase inhibitors will have the ability to influence most, if not all, of these seven mechanisms. Evidence available in the literature indicates that methylation inhibitors do influence these mechanisms, although there was no suggestion to use such inhibitors in a clinical  
10 environment. It is now proposed that various methylation inhibitors alone could be used as antimicrobial agents and, more particularly, that their use in combination with known antimicrobials will reduce or eliminate resistance.

15

The present invention was developed in part from a detailed analysis of the scientific literature and an assimilation of known, but previously unconnected, facts. Certain of the publications in this area are described in  
20 the following sections.

Butler & Gotschlich (1991) reported that antibiotic resistance in *Neisseria gonorrhoeae* had been associated with the acquisition of R plasmids from heterologous  
25 organisms. RSF1010-like plasmids are the first multiresistant plasmid group found in *Neisseria* spp., having resistance not only to sulfonamides and streptomycin but also to ampicillin. They studied the possibility of dissemination of these plasmids into  
30 *N. gonorrhoeae*. It was found that RSF1010 could be mobilized into *N. gonorrhoeae* at a very low frequency, but this frequency increases by greater than 4 orders of magnitude when the plasmid DNA is methylated in vivo by a specific methylase. It was suggested that protection  
35 from digestion against the gonococcal restriction enzymes

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in vitro correlates with a substantial increase in the conjugal frequency.

Stein et al. (1988) demonstrated that transformation  
5 of gonococcal plasmid pFT180 isolated from *E. coli* HB101  
or *N. gonorrhoeae* WR302 into that gonococcal strain PGH3-  
2 required in vitro methylation with a specific  
methylase. In the absence of methylation, a five-orders  
of magnitude decrease in the transformation frequency was  
10 observed.

Caboche & Bachellerie (1977) analyzed ribosomal RNA  
maturation in eukaryotes and observed that the global  
efficiency of the process of rRNA maturation is severely  
15 affected in qualitative terms. The undermethylation  
partially inhibits several stages in the maturation  
pathway and therefore does not block selectively at a  
definite step of processing. Two main alterations are  
observed. First, in the nucleus the life times of the  
20 various undermethylated intermediates of rRNA maturation  
are markedly increased and a significant accumulation of  
these forms is detected by long-term labelling studies.  
Second, the rate of appearance of 32S rRNA into the  
cytoplasm is severely affected in much higher proportions  
25 than the formation of its immediate precursor, nuclear  
28S rRNA, suggesting that the lack of methylation may  
play an important role in the last step of maturation  
(possibly release into the cytoplasm).

30 Although bacteria contain no nuclei, the processing  
of rRNA is surprisingly similar to that of eukaryotes.  
Additionally, fungi and parasites are eukaryotes and  
should behave as described above. It is therefore  
expected that cycloleucine should have some of the same  
35 effects on bacterial rRNA maturation. The present  
invention is thus contemplated to be useful against

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bacteria and other microbial infections, e.g., those caused by yeast and parasitic microbes.

5 Yamaki et al. (1988) and others showed that *dam* methylation is important in the regulation of initiation of DNA replication in *E. coli*. These authors determined that the thermal melting temperature of the *oriC* region is lowered by adenine methylation at GATC sites. It is proposed that *dam* methylation might lead to more  
10 efficient transcription at promoters near *oriC* which may stimulate initiation of DNA replication at *oriC*. Recent studies also reportedly showed reduced frequency of transformation of *oriC* plasmids in *dam* mutants, and poor functioning of *oriC* plasmids derived from a *dam* mutant in  
15 an *in vitro* DNA replication system. This leads the inventors to predict that methylation inhibitors will likely slow down the efficiency transcription and replication of plasmids.

20 Sinefungin and SAH are known to effect DNA and protein methylases from *Streptomyces* and other bacteria and to inhibit the development of various fungi, viruses and parasites (e.g., Barbes et al., (1990)). It has been reported that sinefungin inhibits the methylation of  
25 adenine or guanine to a greater extent than cytosine in tRNA (Vedel & Robert-Gero, 1981; Barbes et al., 1990). It has also been observed that DNA methylases from *S. antibioticus*, a non-sinefungin producer, are noticeably inhibited, but not in the producer *S.*  
30 *incarnatus*. Barbes et al. (1990) suggested that DNA methylation, and possibly RNA methylation, appear to be influenced in non-sinefungin producers. They also suggested that sinefungin could be a useful tool in investigating the physiological processes related to  
35 bacterial methylation, but did not suggest the clinical

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use of sinefungin in combination with antibiotics to reduce antibiotic resistance.

Yebra et al. (1991) reported that sinefungin and derivatives inhibited RNA methyltransferase in *S. antibioticus*, *S. incarnatus* and *S. griseolus*. The structural features of sinefungin that are essential for its inhibitory effect were also identified (Yebra et al., 1991, incorporated herein by reference). The maintenance of such important structural features in analogues intended for use in the present invention is required. When *E. coli* 16S and 23S rRNA were used as a substrate, lower exogenous methylation levels were observed, namely 10% of those obtained with tRNA.

Yebra et al. (1991) speculated further that, due to the instability of mRNA, that it is more conceivable that the modifications effected were on the precursors of mature rRNA and/or tRNA molecules during the process of formation. They also mentioned that sinefungin inhibits sporulation of *S. antibioticus*, but didn't inhibit the synthesis of DNA methyltransferases.

Lorian (1991) described the method of transduction in which bacteria acquire new DNA, including the transfer of resistance genes for  $\beta$ -lactamase and heavy metal-resistance plasmids of staphylococci, originally described in the 1960's and 1970's. These plasmids are packaged by a phage and carried from one cell to another, a process that has been shown to occur in vitro and in vivo.

It is generally accepted according to Lorian, that the most common mechanism of transfer of resistance genes is by conjugation in which cell-to-cell contact is made, and DNA from one cell (the donor) is transferred to

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another cell (the recipient). Therefore, in the latter case, the plasmid is able to mediate its own transfer by conjugation.

5        Razin et al. (1975) showed that methylation of bacteriophage DNA takes place during the last stage of DNA replication. The inhibition of this DNA methylation is accompanied by a corresponding decrease in phage  
10        production, indicating the vital role of the methylation step in the maturation of the virus. The Lorian and Razin papers suggest to the inventors that methylation inhibitors will likely disrupt plasmid/phage mediated antibiotic resistance.

15        Zyskind et al. (1992) indicated that three cellular processes occur during conversion of a mother bacterial cell into daughter cells: DNA replication, cell growth, and cell division. In procaryotic cells, the rate of  
20        replication is controlled not by the rate of chain elongation, but by the rate of initiation at the origin of replication, *oriC*. The authors suggested that one or more GATC sites in the origin region must be fully  
25        methylated for the origin to function in initiation. They concluded that as part of the mechanism, initiation can occur only after methylation of the DNA of the *oriC* region.

Protein carboxymethylase is involved in mediating bacterial chemotaxis (Diliberto et al., 1976; Aswad et  
30        al., 1974). Flagellated bacteria require S-adenosylmethionine's (SAM's) mediated methylation for chemotaxis. Additionally, a rapid reversible methylation of a protein in the cytoplasmic membrane of *E. coli* has been reported to increase substantially during the  
35        chemotactic response. The inventors expect that methylation inhibition would influence these processes to

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the detriment of the organism and that the combination of methylation inhibitors and antimicrobial agents will likely be synergistic.

5        Park et al. (1987) showed that N-methylation of apocytochrome c facilitates the import of this protein into the mitochondria by a specific receptor based mechanism, which further illustrates the central role of methylation. This may be relevant when the present  
10      invention is used against eukaryotic microbes.

      Pandit et al. (1992) indicated that the presence of methylated cytosine residues in the integrated DNA is strongly correlated with the number of plasmid copies.  
15      They observed that *hph* genes heavily methylated contained many plasmid copies in transformants. In contrast, the genes those transformants that contains a single plasmid are not heavily methylated.

## 20      Transmethylation

      Most transmethylation reactions involve the transfer of a donor methyl group from SAM to an acceptor molecule and result also in the formation of  
25      S-adenosylhomocysteine (SAH), which is an inhibitor of adenosylmethionine-dependent methyltransferases. Acceptor molecules in these reactions include protein carboxy groups, lysine groups, basic aminoacyl residues in proteins, phospholipids, nucleic acids and various  
30      small molecules. These reactions play an essential role in specialized biological phenomena, such as chemotaxis, neurosecretion, membrane receptor interactions, DNA modification restriction, gene expression, DNA  
35      transcription, RNA localization, RNA processing and cellular proliferation and differentiation (Lawrence & Robert-Gero, 1993).

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Methylation is known to be important in RNA maturation Salim & Madden (1973). In HeLa cells, the 45S precursor ribosomal RNA is about 13,000 nucleotides long and is processed to form the 18S, 28S and 5.8S rRNAs.

5 Extensive spacer regions are eliminated during the ribosomal RNA processing. Before processing, about 110 methylations, termed early methylation, occur rapidly in the nucleolus on the 45S rRNA. These are 2'-O-ribose methylations, and they occur in molar yields for nearly  
10 all positions. Cleavage then occurs to produce smaller RNAs in which all of the methyl groups are conserved. The 45S goes to 41S which, in turn, splits into two pathways: first, 41S to 32S to 28S rRNA; and second, 41S to 20S to 18S rRNA. Subsequently, during late  
15 maturation, seven base methylations occur.

It is known that inhibition of methylation leads to serious extensive degradation of rRNA in Hela cells (Lhoest & Colson, 1990). Although these authors cited  
20 studies where it appears that under methylation does not play an important role in prokaryotic rRNA maturation, other studies showed that inhibition of methylation by sinefungin in *S.cerevisiae* leads to a decrease in stable 18S rRNA (Li et al., 1985).

25

SAM serves as the methyl donor for numerous methyltransferase reactions (De Clercq, 1987). Effective methylation requires the concerted action of a number of enzymes: methyltransferases, SAH hydrolase,  
30 dihydropteroate synthetase, dihydrofolate reductase, SAM synthetase, methionine synthetase, homocysteine transmethylase, ornithine decarboxylase, arginine decarboxylase and adenosine deaminase.

35 SAM itself is synthesized from ATP and methionine. SAM, which is a product of, and an inhibitor of,



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5 methyltransferase reactions, is generated when transfer of a methyl group from SAM to acceptor substrate occurs. To avoid SAH-mediated inhibition and to allow the methyltransferases to proceed with their action, SAH has to be removed by the enzyme SAH hydrolase. SAH hydrolase catalyzes the reversible hydrolysis of SAH to homocysteine and adenosine. Homocysteine and adenosine, in turn, are product inhibitors of the SAH hydrolase reaction, which means that they have to be removed if SAH hydrolase is to proceed with its catalytic function. This can be achieved by several pathways which involve conversion of homocysteine to methionine, to cysteine or to oxidation products, and conversion of adenosine to inosine by adenosine deaminase.

15 It can thus be seen that effective methylation requires the concerted action of at least three types of enzymes: methyltransferases, SAH hydrolase, adenosine deaminase and several enzymes involving homocysteine. Based on this requirement, the inventors envision certain primary types of methylation inhibitors for use in connection with MLS antibiotics: compounds that inhibit methyltransferases; SAH analogues, acyclic and carbocyclic adenosine analogues that inhibit SAH hydrolase; inhibitors of adenosine deaminase and homocysteine metabolism.

Furthermore, methylation will also be influenced if the synthesis of SAM or methionine is altered. Therefore, inhibition of any enzymes required for the synthesis of SAM, such as SAM synthetase, dihydropteroate synthase, dihydrofolic reductase and homocysteinetransmethylase are contemplated to be effective ways by which to lower the effective concentration of SAM, and thus to decrease the level of methylation reactions. Additionally, compounds that

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inhibit maturation of rRNA subunits and any step in protein translation are also contemplated to be useful, as are compounds that inhibit polyamine synthesis.

5       A large number of methylation inhibitors have been described in the literature and, although none have been proposed for use in conjunction with reducing MLS antibiotic resistance, many such inhibitors have been studied for their ability to affect mRNA and small  
10   molecule methylation. The preparation and use of various compounds and agents that are capable of inhibiting bacterial RNA methylation, synthesis and/or maturation is described further in the detailed examples.

#### 15   **MLS Antibiotics**

         Weisblum (1984) reported that what appeared initially as uncomplicated resistance to erythromycin in gram positive bacteria, actually constituted a resistance  
20   syndrome, dependent upon biochemical modification of the 50S ribosome subunit, which in turn, confers resistance to three chemically distinct classes of antibiotics - the macrolides, lincosamides, and streptogramin type B (MLS) antibiotics. Bacteria become resistant to erythromycin  
25   by single biochemical alteration of the ribosomes, as a result of which, the affinity between the drug and ribosome is reduced. By this single alteration, namely, N<sup>6</sup>-methylation or N<sup>6</sup>,N<sup>6</sup>-dimethylation of adenine in 23S ribosomal RNA, a structural component of the 50S ribosome  
30   subunit, bacteria become more generally resistant to the MLS antibiotics.

         The MLS antibiotics comprise three of at least ten chemical distinct classes that inhibit protein synthesis  
35   by their action on the 50S ribosomal subunit. In view of the apparent functional relationship defined by observed

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co-resistant patterns, these three group of inhibitors were designated collectively as the MLS antibiotics.

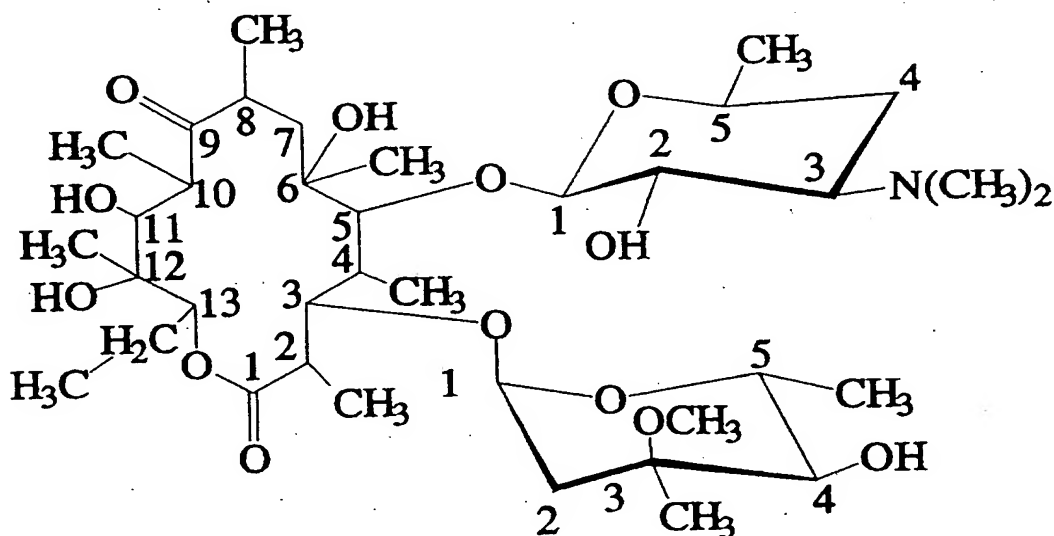
MLS antibiotics are chemically distinct but have a similar mode of action. Their spectrum of activity includes gram-positive cocci (in particular, staphylococci, streptococci, and enterococci) and bacilli and gram negative cocci. These drugs, especially clindamycin, are also potent against anaerobic bacteria. Gram-negative bacilli are usually resistant to MLS antibiotics, but certain enteric bacilli and *Haemophilus* spp. are susceptible to azithromycin. The activities of these antibiotics against *Campylobacter*, *Legionella* and *Chlamydia* spp. are at the origin of the recent renaissance of erythromycin.

Macrolides are composed of a minimum of two amino and/or neutral sugars attached to a lactone ring of variable size. Macrolides that are commercially available or in clinical development can be divided into 14-, 15- and 16-membered lactone ring macrolides. These classes differ in their pharmacokinetics properties and in their responses to bacterial resistance mechanisms. Lincosamides (lincomycin and the more active semisynthetic derivative clindamycin) are alkyl derivatives of proline and are devoid of a lactone ring. Streptogramin antibiotics are used clinically in certain countries, including Belgium and France. They are composed of two factors, A and B (I and II in pristinamycin and M and S in virginiamycin, respectively), that act synergistically and are produced by the same microorganism.

In 1956, a few years after the clinical introduction of erythromycin, resistance of staphylococci to this drug emerged and subsequently spread to France, the United

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Kingdom, and the United States. The MLS cross-resistance phenotype due to modification of the drug rRNA target is widely distributed. Resistance has since been detected in *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Corynebacterium diphtheria*, *Bacteroides* spp., *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Mycoplasma pneumonia*, *Campylobacter* spp. *Propionibacterium* spp. and members of the family *Enterobacteriaceae*. (LeClercq & Courvalin, 1991). The structure of erythromycin A, the proto-typical macrolide is shown below:



In the erythromycin family, three compounds are typically used clinically: erythromycin, azithromycin and clarithromycin (Reese & Betts, 1993). Erythromycin is one of the safest antibiotics in current clinical use. Bacteria known to be susceptible to erythromycin are listed in Table 6 (taken from Lorian, 1991) and in Table 7 (taken from Reese & Betts, 1993). This further serves to indicate the utility of the invention and the wide-

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ranging clinical use for the combinations disclosed herein.

5 Azithromycin a new azolide antibiotic approved for clinical use in 1991. This is a novel 15-member azolide antibiotic. Its structure differs from that of erythromycin in that the lactone ring contains a nitrogen atom. This molecular rearrangement has resulted in a compound with an increased spectrum of activity, yielding  
10 high and sustained tissue antibiotic levels that are much greater than the serum antibiotic levels, and prolonged tissue half-life. This allows for fewer doses during a course of therapy.

15 Clarithromycin is chemically different from erythromycin by having an O-methyl substitution at position 6 of the macrolide ring. Its spectrum of activity is similar to that of erythromycin, except for enhanced *H. influenzae* activity, but it has better  
20 stability properties, including a twice-daily dose regimen.

The mechanism of action of the erythromycin-like antibiotics involves binding to rRNA and prevention of  
25 bacterial protein synthesis. The *ermC* gene confers resistance to erythromycin in gram-positive bacteria by specifying a 23S rRNA methylase that N<sup>6</sup>-methylates or N<sup>6</sup>,N<sup>6</sup>-dimethylates an adenine residue at position 2,058 on the 23S rRNA, thus decreasing ribosomal affinity for  
30 erythromycin at that site (Skinner et al., 1983; Weisblum et al., 1971). The *ermC* expression is induced by erythromycin, and erythromycin binding to unmethylated ribosomes is required for induction. The induction mechanism, termed translational attenuation, has been  
35 intensively studied (Denoya et al., 1986).

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The *ermC* mRNA exists in an inactive conformation in which the ribosome binding site (Shine Dalgarno sequence [CD2]) for methylase synthesis is sequestered by base pairing in a double-stranded region. Stalling of a ribosome under the influence of erythromycin binding during translation of a 19 amino acid leader peptide causes the inactive mRNA structure to open, allowing initiation of methylase synthesis. Translation induction appears to be further regulated by a methylation-mediated feedback loop. Upon induction, the intracellular level of methylase increases, as does the number of methylated ribosomes, with a co-commitment decrease of the ribosomal affinity for erythromycin and prevention of further induction. The overall regulation of *ermC* expression has been summarized by Denoya et al. (1986).

Zalacain & Cundliffe (1990) described methylation of 23S ribosomal RNA due to *carB*, an antibiotic-resistance determinant from the carbomycin producer *Streptomyces thermotolerans*. They showed the *carB* product monomethylates the amino group of the adenosine residue located at position 2058 in 23S rRNA. In contrast, ribosomes from *S. lividans* expressing *ermE*, in which the 23S rRNA is dimethylated at this same position, are highly resistant to macrolides and insensitive to lincosamides. Thakker-Varia et al. (1985) reported that the methylation reaction is affected by a class of methylase, whose genes are typically plasmid- or transposon-associated, and whose synthesis is inducible by erythromycin.

There are apparently no practical proposals in the literature as to how bacterial resistance to MLS antibiotics may be overcome. Although generally applicable to all antimicrobial agents, the present inventors particularly propose the use of one or more

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methylation inhibitors, in conjunction with MLS antibiotics. These will reduce the bacterium's ability to methylate rRNA and allow the antibiotics to bind to their natural rRNA targets, thus inhibiting protein synthesis. Although elegantly simple in hindsight, this represents a novel and important development in anti-microbial strategies.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques proposed by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

#### EXAMPLE I

##### Antibiotic Treatment

Antibiotics such as erythromycin, clindamycin, azithromycin, clarithromycin, vancomycin and the like, may be used clinically at a range of doses. Depending on the circumstances, antimicrobial agents may be employed in oral or parenteral treatment regimens. Appropriate doses are well known to those of skill in the art and are described in various publications, such as (Reese & Betts, 1993; incorporated herein by reference). Table 4 and Table 5 (taken from Reese & Betts, 1993) are included herein to provide ready reference to the currently recommended doses of a variety of antimicrobial agents.

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Following are definitions of terms that are used in Table 4 and Table 5: qid (4 times daily), tid (3 times daily), bid (twice daily), qd (once daily), q4h (every 4 hours around the clock), q6h (every 6 hours around the clock) and q8h (every 8 hours around the clock).

5



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TABLE 4. COMMON ANTIBIOTICS AND USUAL ORAL DOSES

ANTIBIOTIC	DOSAGE
Penicillin V Rugby (generic) V-cillin K	250 mg qid
Dicloxacillin Glenlawn (generic) Dynapen	250 mg qid
Cloxacillin (Tegopen)	250 mg qid
Amoxicillin Rugby (generic) Polymox	250 mg tid
Ampicillin Moore (generic) Polycillin	250 mg qid
Augmentin 250-mg tablets chewables (250 mg) 125-mg (suspension) chewables (125 mg)	tid
Carbenicillin (Geocillin)	382 mg qid (1 tb) 2 tab qid
Cephalexin Rugby (generic) Keflex	250 mg qid
Rugby (generic) Keflex	500 mg qid
Cefadroxil Rugby (generic) Duricef	1 gm bid
Cephradine Rugby (generic) Velosef	250 mg qid
Rugby (generic) Velosef	500 mg qid
Cefaclor Ceclor	250 mg tid
Cefuroxime axetil Ceftin	125 mg bid 250 mg bid 500 mg bid
Cefixime Suprax	400 mg q24h

TABLE 4 (continued)

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ANTIBIOTIC	DOSAGE
Cefprozil Cefzil	250 mg q12h
Loracarbef (Lorabid)	200 mg bid
Cefpodoxime proxetil (Vantin)	200 mg bid
Clindamycin Cleocin	300 mg q8h
TMP/SMZ Bactrim Septra (generic)	1 double- strength bid
Trimethoprim Rugby (generic) Proloprim	100 mg bid
Erythromycin (base) Abbott E-mycin (delayed release)	250 mg qid
Erythromycin stearate Rugby (generic)	250 mg qid
Azithromycin Zithromax	1 g once only 500 mg, day 1, plus 250 mg, day 2-5
Clarithromycin Biaxin	250 mg bid 500 mg bid
Tetracycline hydrochloride Mylan Sumycin 250	250 mg qid
Doxycycline  Lederle (generic) Vibramycin	100 mg qd (with 200-mg initial load)
Vancomycin Vancocin HCl (oral soln/powder)	Capsules 125 mg q6h PO
Metronidazole Rugby (generic) Flagyl	250 mg qid
Norfloxacin Noroxin	400 mg bid

TABLE 4 (continued)

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ANTIBIOTIC	DOSAGE
Ciprofloxacin Cipro	250 mg bid 500 mg bid 750 mg bid
Ofloxacin Floxin	200 mg bid 300 mg bid 400 mg bid
Lomefloxacin Maxaquin	400 mg once qd

TABLE 5. COMMON ANTIBIOTICS AND USUAL PARENTERAL DOSES

ANTIBIOTIC	DOSAGE
Penicillin G Pfizerpen G (Pfizer)	2,400,000 units 12 million units
Oxacillin Prostaphlin (Bristol)	12 g
Nafcillin Nafcil (Bristol)	12 g
Ampicillin Omnipen (Wyeth)	6 g
Ticarcillin Ticar (Beecham)	18 g
Piperacillin Pipracil (Lederle)	18 g 16 g
Mezlocillin Mezlin (Miles)	18 g 16 g
Ticarcillin-clavulanate Timentin (Beecham)	18 g/0.6 g 12 g/0.4 g
Ampicillin-sulbactam Unasyn (Roerig)	6 g 12 g
Cephalothin Keflin (Lilly)	9 g (1.5 g q4h)
Cefazolin Ancef (SKF)	4 g (1 g q6h) 3 g (1 g q8h)
Cefuroxime Zinacef (Glaxo)	6 g 2.25 g (750 mg q8h) 4.5 g (1.5 g q8h)
Cefamandole Mandol (Lilly)	9 g (1.5 g q4h)
Cefoxitin Mefoxin (MSD)	8 g (2 g q6h) 6 g (2 g q8h)
Cefonicid Monicid (SKF)	1 g q12h
Cefotetan Cefotan (Stuart)	2 g q12h
Cefmetazole Zefazone (Upjohn)	2 g q8h
Ceftriaxone Rocephin (Roche)	2 g (2.0 g q24h) 1 g (1.0 g q24h)

TABLE 5 (continued)

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ANTIBIOTIC	DOSAGE
Ceftazidime Fortax (Glaxo) Taxicef (SKF) Tozidime (Lilly)	6 g (2 g q8h)
Cefotaxime Claforan (Hoechst)	2 g q6h 2 g q8h
Cefoperazone Cefobid (Pfizer)	8 g (2 g q6h) 6 g (2 g q8h)
Ceftizoxime Ceftizox (SKF)	(2 g q8h)
Aztreonam Azactam (Squibb)	2 g q8h 1 g q8h
Imipenem Primaxin (MSD)	2000 mg (500 mg 16h)
Gentamicin Garamycin (Schering) (generic) (Elkins-Sinn)	360 mg (1.5 mg/kg q8h for an 80-kg patient)
Tobramycin Nebcin (Dista)	360 mg (1.5 mg/kg q8h for an 80-kg patient)
Amikacin Amikin (Bristol)	1200 mg (7.5 mg/kg q12h for an 80-kg patient)
Clindamycin Cleocin (Upjohn)	2400 mg (600 mg q6h) 2700 mg (900 mg q8h) 1800 mg (600 mg q8h)
Chloramphenicol Chloromycetin (P/D)	4 g (1 g q6h)
TMP/SMZ Septra (Burroughs Wellcom)	1400 mg TMP (5 mg TMP/kg q6h for a 70-kg patient) 700 mg TMP (5 mg TMP/kg q12h for a 70-kg patient)
Erythromycin Erythromycin (Elkins-Sinn)	2000 mg (500 mg q6h)

TABLE 5 (continued)

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ANTIBIOTIC	DOSAGE
Doxycycline Vibramycin (Pfizer)	200 mg (100 mg q12h)
Vancomycin Vancocin (Lilly)	2000 mg (500 mg q6h)
Metronidazole (generic) (Elkins-Sinn)	2000 mg (500 mg q6h)
Ciprofloxacin Cipro	200 mg q12h 400 mg q12h
Pentamidine Pentam (LyphoMed)	280 mg (4 mg/kg q24h for a 70-kg patient)

The effectiveness of erythromycin and lincomycin against a wide variety of organisms is shown in Table 6 (taken from Lorian, 1991). Table 7 (taken from Reese & Betts, 1993), is provided as a comparison of certain common MLS antibiotics. The data presented in the tables of the present specification is another tool to enable the straightforward comparison of raw data with accepted clinical practice and to allow the determination of appropriate doses of combined agents for clinical use.

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TABLE 6  
SUSCEPTIBILITY TO ANTIBIOTICS

## ERYTHROMYCIN

Species	(n)	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>Bacillus</i> spp.	20	0.03-2	0.25	2
<i>Bacteroides fragilis</i>	97	≤0.25-16	1	8
<i>Bordetella bronchiseptica</i>	11	4-32	8	32
<i>Bordetella parapertussis</i>	46	≤0.125-4	0.25	0.25
<i>Bordetella pertussis</i>	32	1-0.5	0.25	0.25
<i>Bordetella pertussis</i>	75	≤0.125-0.5	≤0.125	≤0.125
<i>Borrelia burgdorferi</i>	10	0.03-0.125	0.03	0.06
<i>Branhamella</i> (Moraxella) <i>catarrhalis</i>	20	≤0.125-0.5	0.25	0.25
<i>Branhamella</i> (Moraxella) <i>catarrhalis</i>	20	0.125-0.5	0.25	1
<i>Branhamella</i> (Moraxella) <i>catarrhalis</i> (non $\beta$ -lactamase producer)	40	≤0.06-0.5	0.25	0.5
<i>Branhamella</i> (Moraxella) <i>catarrhalis</i> (non $\beta$ -lactamase producer)	13	0.03-0.125	0.06	0.06
<i>Branhamella</i> (Moraxella) <i>catarrhalis</i> (non $\beta$ -lactamase producer)	14	≤0.06-1	0.125	1
<i>Branhamella</i> (Moraxella) <i>catarrhalis</i> (non $\beta$ -lactamase producer)	16	0.015-1	0.06	0.25

TABLE 6 (continued)

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Species	(n)	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>Branhamella</i> ( <i>Moraxella</i> ) <i>catarrhalis</i> ( $\beta$ - lactamase producer)	47	$\leq 0.06-1$	0.25	0.5
<i>Branhamella</i> ( <i>Moraxella</i> ) <i>catarrhalis</i> ( $\beta$ - lactamase producer)	58	0.03-0.25	0.125	0.125
<i>Branhamella</i> ( <i>Moraxella</i> ) <i>catarrhalis</i> ( $\beta$ - lactamase producer)	160	$\leq 0.06-8$	0.25	0.5
<i>Branhamella</i> ( <i>Moraxella</i> ) <i>catarrhalis</i> ( $\beta$ - lactamase producer)	35	0.03- 0.125	0.06	0.06
<i>Campylobacter</i> <i>jejuni</i>	25	0.5-8	1	4
<i>Campylobacter</i> <i>jejuni</i>	16	0.125-4	0.25	2
<i>Campylobacter</i> <i>pylori</i>	56	0.25-16	0.5	1
<i>Campylobacter</i> <i>pylori</i>	13	0.125- 0.25	0.125	0.25
<i>Corynebacterium JK</i>	102	0.5- $\geq 128$	$\geq 128$	$\geq 128$
<i>Corynebacterium JK</i>	19	0.125- $\geq 64$	2	$\geq 64$
<i>Enterococcus</i> <i>faecalis</i>	26	1- $\geq 64$	1	4
<i>Enterococcus</i> <i>faecalis</i>	50	0.06- $\geq 64$	4	$\geq 64$
<i>Enterococcus</i> <i>faecalis</i>	86	0.125- $\geq 64$	1	$\geq 64$
<i>Enterococcus</i> <i>faecalis</i>	97	0.125-128	2	128
<i>Enterococcus</i> <i>faecium</i>	14	$\leq 0.06$ - $\geq 64$	1	$\geq 64$
<i>Enterococcus</i> spp.	35	$\leq 0.06$ - $\geq 32$	2	$\geq 32$
<i>Haemophilus ducreyi</i>	122	?-0.125	0.004	0.06
<i>Haemophilus</i> <i>influenzae</i>	145	0.5-8	2	2



TABLE 6 (continued)

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Species	(n)	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>Haemophilus influenzae</i>	97	0.25-≥16	1	4
<i>Haemophilus influenzae</i> (non $\beta$ -lactamase producer)	22	0.125-8	2	4
<i>Haemophilus influenzae</i> (non $\beta$ -lactamase producer)	137	≤0.06-≥8	4	8
<i>Haemophilus influenzae</i> ( $\beta$ -lactamase producer)	46	≤0.06-≤8	4	8
<i>Haemophilus influenzae</i> ( $\beta$ -lactamase producer)	17	0.25-4	2	4
<i>Haemophilus influenzae</i> (penicillin susceptible)	22	0.25-16	8	16
<i>Haemophilus influenzae</i> (penicillin resistant)	20	8-16	8	16
<i>Haemophilus parainfluenzae</i>	13	0.5-8	2	4
<i>Legionella</i> spp.	23	0.03-0.25	0.125	0.25
<i>Legionella pneumophila</i>	31	≤0.0075-0.25	0.06	0.125
<i>Legionella pneumophila</i>	48	0.03-2	0.25	0.5
<i>Legionella pneumophila</i>	25	0.125-1	0.25	1
<i>Listeria monocytogenes</i>	13	0.5-1	0.5	0.5
<i>Listeria monocytogenes</i>	16	0.125-2	0.25	1
<i>Listeria monocytogenes</i>	65	≤0.06-≥32	0.125	32

TABLE 6 (continued)

- 60 -

Species	(n)	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>Mycoplasma hominis</i>	26	≥128	≥128	≥128
<i>Mycoplasma hominis</i>	20	≥256	≥256	≥256
<i>Mycoplasma pneumoniae</i>	10	≤0.06-8	≤0.06	≤0.06
<i>Mycoplasma pneumoniae</i>	14	≤0.004-0.03	≤0.004	≤0.004
<i>Neisseria gonorrhoeae</i>	19	0.0075-8	0.25	1
<i>Neisseria gonorrhoeae</i> (non β-lactamase producer)	73	0.015-4	0.25	2
<i>Neisseria gonorrhoeae</i> (non β-lactamase producer)	78	0.03-2	0.25	1
<i>Neisseria gonorrhoeae</i> (β-lactamase producer)	12	0.03-4	0.5	2
<i>Neisseria gonorrhoeae</i> (β-lactamase producer)	17	1-4	2	4
<i>Neisseria meningitidis</i>	19	0.5-8	1	8
<i>Nocardia asteroides</i>	78	≤0.25-≥8	≥8	≥8
<i>Staphylococcus aureus</i>	44	0.125-1	0.125	0.5
<i>Staphylococcus aureus</i>	100	0.25-128	0.5	4
<i>Staphylococcus aureus</i> (penicillin susceptible)	20	0.125-0.5	0.5	0.5
<i>Staphylococcus aureus</i> (penicillin susceptible)	35	≤0.06-≥32	0.25	0.5

TABLE 6 (continued)

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Species	(n)	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>Staphylococcus aureus</i> (penicillin resistant)	35	0.25- $\geq 32$	0.25	$\geq 32$
<i>Staphylococcus aureus</i> (methicillin susceptible)	28	0.125-1	0.25	0.5
<i>Staphylococcus aureus</i> (methicillin susceptible)	97	$\leq 0.125$ - $\geq 64$	0.25	$\geq 64$
<i>Staphylococcus aureus</i> (methicillin susceptible)	20	0.125-1	0.5	0.5
<i>Staphylococcus aureus</i> (methicillin resistant)	17	0.5- $\geq 128$	128	128
<i>Staphylococcus aureus</i> (methicillin resistant)	15	$\geq 64$	$\geq 64$	$\geq 64$
<i>Staphylococcus aureus</i> (methicillin resistant)	20	$\geq 64$	$\geq 64$	$\geq 64$
<i>Staphylococcus aureus</i> (methicillin resistant)	30	$\leq 0.06$ - $\geq 32$	$\geq 32$	$\geq 32$
<i>Staphylococcus coagulase</i> $\phi$	10	0.125-4	0.25	2
<i>Staphylococcus coagulase</i> $\phi$	100	0.125- $\geq 64$	0.25	$\geq 64$
<i>Staphylococcus coagulase</i> $\phi$ (non $\beta$ -lactamase producer)	12	0.03-8	0.125	0.25
<i>Staphylococcus coagulase</i> $\phi$ ( $\beta$ -lactamase producer)	38	0.06-16	0.125	4

TABLE 6 (continued)

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Species	(n)	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>Staphylococcus epidermidis</i>	50	0.125-≥64	≥64	≥64
<i>Staphylococcus haemolyticus</i>	20	0.125-≥64	≥64	≥64
<i>Staphylococcus hominis</i>	20	0.125-≥64	≥64	≥64
<i>Streptococcus agalactiae</i>	20	0.03-0.25	0.03	0.125
<i>Streptococcus agalactiae</i>	34	0.015-0.06	0.03	0.03
<i>Streptococcus pneumoniae</i>	58	0.03-0.25	0.06	0.125
<i>Streptococcus pneumoniae</i>	91	0.125-4	0.125	0.125
<i>Streptococcus pneumoniae</i>	50	0.015-0.06	0.03	0.03
<i>Streptococcus pneumoniae</i>	16	≤0.03-0.125	0.06	0.125
<i>Streptococcus pneumoniae</i>	26	0.015-0.25	0.03	0.06
<i>Streptococcus pneumoniae</i>	50	0.03-0.125	0.06	0.06
<i>Streptococcus pyogenes</i>	19	0.03-0.25	0.06	0.125
<i>Streptococcus pyogenes</i>	20	≤0.03-0.25	0.06	0.125
<i>Streptococcus pyogenes</i>	33	0.015-0.03	0.03	0.03
<i>Streptococcus pyogenes</i>	20	≤0.06-≥32	0.125	≥32
<i>Streptococcus</i> spp.	22	0.015-0.25	0.03	0.06
<i>Streptococcus</i> spp.	107	0.004-2	0.03	1
<i>Ureaplasma urealyticum</i>	28	0.015-≥256	2	≥256
<i>Ureaplasma urealyticum</i>	19	8-≥128	16	32

TABLE 6 (continued)

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## LINCOMYCIN

Species	(n)	Range	MIC <sub>50</sub>	MIC <sub>90</sub>
<i>Mycoplasma hominis</i>	28	0.5-16	2	4
<i>Mycoplasma pneumoniae</i>	11	2-32	8	32
<i>Staphylococcus aureus</i>	100	0.5-512	1	1
<i>Ureaplasma urealyticum</i>	19	64-≥128	≥128	≥128

TABLE 7. IN VITRO ACTIVITY AND COMPARISON OF SELECTED MACROLIDE ANTIBIOTICS

Organism	Azithromycin MIC <sub>90</sub>	Clarithromycin MIC <sub>90</sub>	Erythromycin MIC <sub>90</sub>
<b>Gram-positive aerobes</b>			
<i>Staphylococcus aureus</i> Methicillin susceptible Methicillin resistant	1.0 <sup>a</sup> >128.0	0.12-0.24 <sup>a</sup> >128.0	0.25-0.50 >128.0
<i>Streptococcus pyogenes</i> (group A) Erythromycin susceptible	0.12 <sup>a</sup>	0.015 <sup>a</sup>	0.03 <sup>a</sup>
<i>Streptococcus pneumoniae</i>	0.12 <sup>a</sup>	0.015 <sup>a</sup>	0.03-1.0 <sup>a</sup>
<i>Streptococcus agalactiae</i> (group B)	0.5 <sup>a</sup>	0.03-0.25 <sup>a</sup>	0.03-0.25 <sup>a</sup>
<i>Streptococcus bovis</i>	0.25		
<i>Enterococci</i> (group D) <i>E. faecalis</i> (Erythromycin susceptible) (Erythromycin resistant)	8.0 >64.0		
<i>E. faecium</i>			
<i>Viridans streptococci</i>	16.0	0.03	0.06
Coagulase-negative staphylococci Methicillin resistant Methicillin susceptible	>128.0		
<i>Listeria monocytogenes</i>	4.0	0.12-2.0	0.5-2.0

TABLE 7 (continued)

Organism	Azithromycin MIC <sub>90</sub>	Clarithromycin MIC <sub>90</sub>	Erythromycin MIC <sub>90</sub>
<b>Gram-negative aerobes</b>			
<i>Haemophilus influenzae</i>	1.0	2.0-16.0	4.0-8.0
<i>Moraxella catarrhalis</i>	0.5	0.25-1.0	0.25-2.0
<i>Neisseria gonorrhoeae</i>	0.15	0.25-2.0	0.25-2.0
<i>N. meningitidis</i>	0.12	--	0.4-1.6
<i>Pasteurella multocida</i>	0.5	--	--
<i>Bordetella pertussis</i>	0.12	--	0.3-1.6
<i>Legionella pneumophila</i>	0.25-2.0	0.25	1.0-2.0
<i>Campylobacter species</i>	0.25	1.0-8.0	0.2->50.0
<b>Anaerobes</b>			
Gram-positive cocci (peptococcus, peptostreptococcus)	2.0	4.0->32.0	2.0->32.0
<i>Bacteroides fragilis</i>	8.0	2.0-8.0	4.0-32.0
<i>Clostridium perfringens</i>	1.0	0.5-2.0	1.0
<b>Miscellaneous</b>			
<i>Chlamydia pneumoniae</i> (formerly TWAR)	0.12-0.25-1.0	0.007	0.065-1.0
<i>Chlamydia trachomatis</i>	0.12-0.25	0.06-0.125	0.12-0.25
<i>Mycoplasma pneumoniae</i>	0.001	0.008-0.03	0.004

<sup>a</sup> Erythromycin-resistant organisms will be resistant to erythromycin alone.

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Using the strategy of the present invention, it also possible to use other antimicrobial agents in combination with the MLS antibiotics and inhibitors described herein. The use of antibiotic combinations is well established, for example, see Eliopoulous & Moellering (1991; incorporated herein by reference). Combinations are used primarily for two reasons: to provide broad-spectrum coverage in patients that are seriously ill and who may be come septicemic and, less frequently, because an identified organism is resistant to traditional doses of single antibiotics, but which the antibiotic combination may give the desired antimicrobial effect. Here it should be noted that the reversal of MLS antibiotic resistance is not discussed in (Eliopoulous & Moellering, 1991), highlighting the need for a new development in this area.

Mechanistic considerations are not important to practicing the invention, with the appropriate antibiotic doses and times for administration being generally known to those practicing in this area (e.g., defined doses at two, three or four times per day). However, the inventors did consider the underlying mechanisms in choosing certain currently preferred modes of administration in regard to MLS antibiotics and methylation inhibitors.

The inventors reasoned that bacterial cells generally contain  $10^3$ - $10^4$  ribosomes, that the induction of methylases reach a steady state of about  $10^3$ - $10^4$  molecules in about 2-3 hours, and that induction stabilizes the erm mRNAs half life from 2-3 minutes to 45 minutes (Denoya et al., 1986). Additionally it has been shown that the first resistant cells appear in about 20 minutes. From the known rate of peptidyl transferase catalysis (Devlin, 1986) and the rate of rRNA



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methylation, it is clear that inductive methylase synthesis occurs rapidly, and it is estimated that most of the ribosomes are methylated within one generation.

5       Following the induction of methylase synthesis, resistance to erythromycin rapidly occurs. Theoretically, principles suggest that to reduce or reverse erythromycin resistance, the best treatment regimen will be when (a) the induction of the synthesis  
10       of the methylase is prevented and/or (b) when methylation of the rRNA is prevented. Both of these can be achieved in a number of ways, using appropriate inhibitors, e.g., where the concentration of the methylating agent SAM is decreased. A preferred treatment will involve the pre-  
15       administration of an inhibitor for a given time period sufficient to decrease both protein synthesis and the concentration of SAM, then followed by the administration of the antimicrobial alone or in combination with more of the same or further inhibitor(s).

20

## EXAMPLE II

### Evaluation of Antibiotic Susceptibility

#### Disk Susceptibility Test

25

To more precisely define appropriate doses of inhibitors, or second agents, for use with MLS antibiotics, the inventors contemplate using a disk susceptibility test. Disk susceptibility testing is  
30       widely used in clinical laboratories (Acar & Goldstein, 1991; Shungu, 1991). The principle of these methods involves the use of a constant concentration of an antibiotic within a reservoir in the agar that is used to culture the organisms of interest. The susceptibility of  
35       the organism to the antibiotic is indicated by a clear zone of inhibition around the reservoir. The diameter of

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the zone of inhibition is proportional to the susceptibility of the organisms in question. The diameter of the clear zone of bacteria in the presence of antibiotics is known at the minimum inhibitory concentration (MIC), therefore bacterial strains resistant to an antibiotic can be identified quite easily from the diameter of the clear zone; resistant bacteria having a small clear zone diameter. The diffusion technique developed by Bauer et al. (1966) is the technique also recommended by the FDA and has been the technique most widely used in the clinical laboratory. This method is endorsed by the WHO and the National Committee for Clinical Laboratory Standards (NCCLS, see NCCLS Document, 1993).

15

The effect of more than one antibiotic, or other agent, on a bacterium is also easily examined with agar disk susceptibility testing. In one such example for use with the present invention, one would use two disks, one each impregnated with an MLS antibiotic, and the other with an inhibitor of RNA methylation. An increased clear zone will be observed where the antibiotic and second agent interact to exert their combined effect.

## 25 Checkerboard Method

The checkerboard (or chessboard) method may also be used to assess antimicrobial combinations. The term "checkerboard" refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested in concentrations equal to, above, and below their minimal inhibitory concentrations (MICs). Also included is a row (or column) of tubes (or microtiter wells) without any antimicrobial for each drug. Thus, (compound A), being diluted along the x-axis, and (compound B), being diluted on the y-axis,

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results in squares on a checkerboard containing a unique combination of the two compounds being tested.

This technique may be performed with liquid or semisolid (agar) media, with microtiter trays rather than racks or test tubes, using other than 2-fold dilutions, using more than two drugs, and with organisms other than bacteria. In the agar dilution method, the principle of the technique is the same, but it is adapted for use in an agar dilution system, which is advantageous when a large number of strains are to be tested against a limited number of antibiotic combinations. Microtiter trays are often preferred because they are less cumbersome and require smaller quantities of antimicrobials and broth.

After conducting a checkerboard analysis, an isobologram is constructed as follows: For each concentration along the x-axis (plotted as the x-coordinate), the lowest concentration of the compound diluted along the y-axis that inhibits growth in the column of tubes is taken as the y-coordinate of this plot. An isobologram is constructed by connecting the series of coordinate points generated for each drug combination.

In interpreting the results, the critical question is usually whether an apparently synergistic combination is significantly below the additive line of the isobologram. Because the margin of error in these studies is  $\pm 1$  dilution, a combination should be at least 2 dilutions below the additive line of the isobologram to be significantly synergistic, or at least 2 dilutions above the additive line to be significantly antagonistic.

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### Killing Curves

Killing curves (time-kill curves, time-kill plots) may also be used. In contrast to the checkerboard, which typically provides only inhibitory data, the killing curve technique measures the microbicidal activity of the combination being tested. For this reason, it is advantageous for clinical situations in which bactericidal therapy is desirable. The other major advantage of killing curves over the checkerboard is that they provide a dynamic picture of antimicrobial action and interaction over time (based on serial colony counts), as opposed to the checkerboard, which is usually examined only once (after 16 to 24 hours or incubation).

When the colony counts in a killing curve study have been determined, the results are plotted and interpreted by the effect of the combination in comparison with the most active single drug alone. Results from bacteriostatic tests are interpreted as follows: Drug combinations are considered synergistic if inhibition occurs with concentrations at or below the curve described by the original MIC of each single drug and  $\frac{1}{2}$  the MIC for both drugs. When an organism is sensitive to the combination at concentrations  $\geq 2$ x the original MIC of each single agent, the antibacterials are designated "antagonistic." The points that fall between synergy and antagonism are defined as additive or "indifferent."

### Serum Bactericidal Testing

The serum bactericidal titer may be determined and used as an estimate of the activity of antimicrobial combinations in patients receiving them. The advantage of this approach is that it measures the activity of antimicrobial concentrations that are achievable *in vivo*.

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### Kinetic Spectrophotometric Methods

Spectrophotometric methods provide both a more  
5 kinetic view of antimicrobial interaction and a more  
quantitative analysis of the dose-response relationship.  
In these methods, one can plot growth curves for multiple  
cultures simultaneously and examine the effects of  
different antimicrobials and antimicrobial combinations  
10 on the growth-rate constants of those cultures. The  
expected 50% inhibitory concentration ( $ED_{50}$ ) of one  
compound can be compared to that in combination with a  
steadily increasing series of concentrations of a second  
compound.

### Paper Strip Diffusion

In the paper strip diffusion method, using  
cellophane or membrane filter transfer, filter paper  
20 strips are soaked in antimicrobial solution and placed at  
right angles to one another on an agar plate. After  
incubation, the filter paper strips are removed, leaving  
behind the compounds that have diffused into the agar  
medium. Following removal of the filter paper strips, a  
25 transferable material (cellophane or a filter membrane)  
that will permit diffusion of the two compounds now  
contained on the agar plate is placed on the agar surface  
and inoculated with a suspension of the organism to be  
tested. After incubation, the transferable material is  
30 removed from the agar plate containing the compounds and  
transferred to another agar plate without antimicrobials.  
Following an additional incubation, the growth pattern is  
examined.

35 Additive or indifferent combinations show no  
enhancement of their zones of inhibition at the former

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junction of the filter papers. Synergistic combinations show enhanced inhibition and may even demonstrate activity at this point, even when the organism is resistant to both compounds separately.

5

The methods of sections 2-6 above are even more fully described in Lorian (1991, pages 432-449).

#### Animal Tests for Activity and Toxicity

10

*In vitro* tests are useful as the starting point in the development of new drugs and combinations for use in treatment. However, no such drug or combination of agents should then be administered to a human patient solely on the basis of *in vitro* activity. This invention provides inhibitory compounds for use in combination with antimicrobials, which compounds have been selected on the basis of reported functional characteristics. As no such inhibitory compound is selected randomly, it is expected that the correlation between *in vitro* and *in vivo* data will be high.

20

However, the correlation between the strength of the *in vitro* and *in vivo* activities of a compound may not be linear. Therefore, certain compounds with high *in vitro* activity may not prove to be the most advantageous for *in vivo* use. This is not a drawback to the present invention, but simply reflects a checkpoint that is known to exist in all antimicrobial testing. Lorian (1991) pointed out that *in vitro* and *in vivo* tests differ in their general characteristics and specific variables. Consequently, discrepancies are likely to occur. However, they may be understood and interpreted correctly if the limitations of the test systems are understood.

35

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As an example, Lorian (1991) discussed a case where discrepancies between *in vitro* and *in vivo* results could be resolved by using a concentration of drug *in vivo* that resulted in plasma levels of the antibiotic similar to those used in the *in vitro* evaluation. Thus, while *in vitro* evaluations such as those used by the inventors serve as an excellent indicator of *in vivo* antimicrobial potential, it is well understood in the art that optimizing an *in vivo* dose often takes more than a direct application of the *in vitro* data. Extrapolation of data to account for variable parameters such as blood volume, tissue accessibility and plasma half life is routinely practiced in the art.

Part of the practice of developing the *in vitro* data for *in vivo* clinical use involves studies in animal models. Lorian (1991) indicated that animal studies with antibiotics are important for suggesting appropriate indications, determining potential toxicity problems and providing insight into the pharmacokinetic properties of new agents in relation to those of known agents.

In evaluating any new antimicrobial or combination thereof, it is well known that the doses necessary to control the disease do not produce toxic reactions worse than those of the disease. Thus, the relationship between toxicity and efficacy must be ascertained. This relationship is known as the therapeutic index. The larger this index, the less likely it is that the drug or combination will cause toxic effects when used clinically. An index of 10 is often regarded as a safe minimum for an antibacterial, and this guideline can be used in the present invention in combination with the data given herein. In experimental studies, the index is usually calculated as the ratio of the dose that kills

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50% of the animals ( $LD_{50}$ ) to the dose that protects 50% of animals ( $PD_{50}$ ).

In analyzing the amount of drug to be administered during animal tests, the maximum tolerated dose of a substance should be determined (using the most suitable route of administration). Before use in the treatment of infected animals, the maximum tolerated dose is determined by administering single injections of the substance to groups of animals by the oral, subcutaneous, or intraperitoneal routes. The animals are then observed for survival for periods of 24 hours to 7 days. Such acute toxicity studies establish for each route the 100% toxic dose ( $LD_{100}$ ), the 50% lethal dose ( $LD_{50}$ ), and the maximum tolerated dose ( $LD_0$ ) at which all animals survive. Experience has allowed the formulation of the rule that approximately 1/5 of the maximum tolerated dose of a substance will be tolerated when treatments are given once daily for 5 days or longer. For 1 to 3 days of treatment, 1/2 the maximum dose can usually be given.

In addition to obtaining initial information on toxicity, some information on absorbability may also be obtained. For example, if a substance is tolerated at 1000 mg/kg when given orally, but toxic when given at a dose of 50 mg/kg intraperitoneally or intravenously, the lack of toxicity by the oral route probably reflects poor oral absorption.

Interspecies variation in response to drugs is known to occur and this possibility must be considered when predicting the effect of the drug in man. If several species of animals are used for the toxicity studies, and the same toxic effects occur in all the species, then it is likely that these effects will also occur in man. Conversely, if no toxic effects are observed in any



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species of animal, except with unrealistic doses, then none are likely to occur with therapeutic doses used in man. The animals most commonly used in toxicity studies are the mouse, rat, rabbit, dog, and monkey. By  
5 utilizing animal models known in the art, as exemplified by those described herein, the combinations of the present invention can readily be developed for use in the clinical environment.

10 The inhibitors and antibiotic/inhibitor combinations identified by the inventors should therefore be evaluated in vivo in small animals in a fashion similar to that described by Lorian for new antibiotics (Lorian, 1991, pp. 746-786, incorporated herein by reference). It is  
15 important to note that since this invention generally concerns new and surprising combinations of known compounds, the testing needed will likely be less vigorous than that needed with a completely new drug. The toxicity data known for any individual drugs may be  
20 used as starting guidelines, and combined doses below such levels used in studies to confirm the advantages of the proposed combinations.

As described by Lorian (1991), animal models most  
25 frequently used in the evaluation of antibacterials may be categorized as basic screening, ex vivo, monoparametric, and discriminative. The ex vivo and monoparametric models are used to measure specific variables (e.g., dosage schedule, serum binding, or  
30 penetration into extravascular spaces). The discriminative systems are employed to differentiate the new agents from related or unrelated active agents. For preliminary evaluation of new agents and combinations, as in this invention, the basic screening system is usually  
35 employed.

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Screening models involve simple one-step infections, simple techniques and schedules of treatment, short-duration experiments, reproducible courses of infection, simple evaluation (all-or-nothing models), economy of  
5 test drugs, and low costs. These requirements are best met by the mouse protection test, which is the most widely used in vivo screening model in antibacterial research. The mouse protection test is suitable for determining efficacy and toxicity of new antibacterials  
10 and it can indicate whether a drug or new combination is likely to be active orally or parenterally. The manner of conducting such tests will be well known to those of skill in the art in light of the present disclosure and the guidelines set forth below.

15

In a typical mouse protection test, the primary host used is the Swiss albino mouse. This is because there is a good correlation between clinical response to an antimicrobial and activity in the mouse; large numbers  
20 are easily obtained at relatively small cost per unit; and because an outbred strain of mice provides a heterogeneous population, which allows for immunological and other host factor variations.

25

In developing an experimental mouse model for in vivo testing, it is desirable to use human pathogens whenever possible. It is also desirable to infect with strains of microorganisms that are sufficiently virulent so that conditioning procedures to lower the host's  
30 resistance are unnecessary. When reproducible infections cannot be achieved by inoculation of the organisms alone, adjuvant stressing measures can be used to lower the host's resistance. In the case of infections with *Staphylococcus aureus*, a commonly employed measure is to  
35 suspend the organisms in 3 to 10% hog gastric mucin and

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to administer 0.5 ml amounts by the intraperitoneal route.

5 In preparing a new agent for testing, the stability, degree of solubility and/or the means by which a uniform suspension may be prepared should be considered. If the antibacterial is insoluble and is to be administered by the oral route, it will generally be prepared in a solution containing 1% carboxymethylcellulose. If an insoluble agent or combination is to be administered by the subcutaneous or intraperitoneal route, a suspension in water is prepared by sonication or homogenization. If these methods fail to produce a uniform suspension, the material is dissolved in DMSO or ethanol. The resulting solution is then diluted to a final concentration of less than 10% DMSO or ethanol, since higher concentrations of these solvents are lethal to mice. For treatment, antibacterials should be freshly prepared each day.

20 In order to obtain reproducible infections, it is necessary to determine the degree of virulence of each strain to be studied. The lowest dilution of the organisms at which all the animals die is defined as the minimum lethal dose (MLD). Alternatively, in place of MLD one can use multiples of the challenge dose of organisms that kills 50% of the animals ( $LD_{50}$ ). After the animals are infected and treated, they are observed over a fixed period and the number of survivors is recorded. The 50% protective dose ( $PD_{50}$ ) is calculated in mg/kg. The 50% protective dose is the same as the 50% curative dose ( $CD_{50}$ ) or 50% effective dose ( $ED_{50}$ ). By altering the treatment route or schedule, differences in activity can be demonstrated. In addition, the relative efficacy of oral and subcutaneous administration of a substance can be compared.

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The mouse protection test has been studied in great detail, and evaluation of antibacterials using this method has produced a number of clinically effective agents (Grunberg & Cleeland, 1977). Various studies have  
5 shown that using the mouse protection test the PD<sub>50</sub> values generally fall into the same ranges as the clinical doses measured in mg/kg. However, this is not an indication that the doses in the mouse models should be exactly the doses employed in the clinical management  
10 of infections. Certainly humans are not treated on the basis of achieving only 50% survival. In spite of this, there is a reasonable overall correlation between the results obtained in the mouse model and clinical effectiveness.

15

Many agents that are not active against systemic infections are active against local infections when applied at the site of infection. Different techniques for producing local infections have been described, with  
20 the basic principle being the induction of a localized infection that is treated by application of the desired agent to the infected site. Antibiotics and combinations known to be inactive when given orally or subcutaneously  
- have been shown to be active when infiltrated into the  
25 site of local infection, as exemplified by the activity of mycin against gram-positive organisms and two gram negative strains (Grunberg et al., 1967).

In the mouse protection tests described above, the  
30 infections are primarily of short duration, unnatural and overwhelming or self-limiting. A number of specific infections in a variety of animal hosts have been utilized to mimic human infections more precisely and will be known to those of skill in the art. Examples  
35 that appear to be reasonably faithful to the human infection are pyelonephritis in the rat and meningitis,

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endocarditis and osteomyelitis in the rabbit. In a number of other experimental infections, the targeting of infection to specific organs, while not as close in mimicking the human situation (e.g., meningitis and pneumonia in mice), provide useful models for predicting the selectivity of new agents and combinations.

The following is a description of certain exemplary discriminative animal models that may be of use in connection with the present invention. For most of the discriminative animal model systems it appears that the agents shown to be very effective experimentally are generally useful clinically (Ernst & Sande, 1981).

A simple model for the formation of intraperitoneal abscesses in mice is available that offers opportunities for studying various aspects of staphylococcal host-parasite interactions occurring in localized lesions. As an example of the formation of abscesses by anaerobes, a model of subcutaneous abscesses in mice caused by *Bacteroides fragilis* has also been described.

The thigh lesion model provides a nonlethal experimental infection to evaluate the effectiveness of an antimicrobial combination and allows the measurement of drug-pathogen interaction and drug pharmacokinetics in the infected host. If the animals are made neutropenic, then the thigh model becomes an excellent system for measuring the drug-microorganism interaction with most of the host defense system eliminated (Vogelman et al., 1988).

Regarding respiratory tract infections, such as pneumonia, various models are available. For example, in the mouse model of pneumonia, disease is induced in mice by intranasal instillation of 3 drops of an undiluted

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overnight culture of *Streptococcus pneumoniae* 6301 in trypticase soy broth containing 5% goat serum (Beskid et al., 1981). A useful model for the production of pneumococcal pneumonia in rats has been described by  
5 Ansfield et al. (1977). For the detection of antitubercular drugs, a number of mice systems have been described. An infection in guinea pigs useful for testing new antitubercular agents is also available. In this model, *M. tuberculosis* H37Rv, whether inoculated by  
10 the subcutaneous or intramuscular routes, produces a progressive, predictable disease.

Models for experimental acute urinary tract infections are commonly used to evaluate antibacterials.  
15 In mice, these infections produce either hematogenous or ascending infections, depending on whether the inoculum is administered intravenously or intravesically, with or without the addition of a foreign body. In the mouse pyelonephritis model, the infected animals, in most  
20 instances, either die or heal spontaneously. Consequently, treatment has to be started shortly after infection in order to alter the course of disease. For chronic pyelonephritis, agents are sought that will control the disease after the pathological damage has  
25 occurred. An infection in rats which stimulates chronic *E. coli* nephritis has been described in Ryan (1976).

A number of systems for studying the effect of antibacterials in experimentally induced meningitis have  
30 been reported. A simple lethal mouse model to produce pneumococcal meningitis is available (Tsai et al., 1975). For induction of meningitis, Swiss albino mice were subjected to strains of *S. pneumoniae* virulent for mice by the intraperitoneal route. A model for the induction  
35 of *H. influenzae* b meningitis in infant rats, which appears to be both simple and reproducible, has also been

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described. An excellent rabbit model used to study the activity of antibacterials in the therapy of experimental meningitis has been described using New Zealand white rabbits (Dacey & Sande, 1974; Scheld et al., 1978).

5

The method described by Garrison & Freedman (1970) for inducing experimental endocarditis in rabbits has been well studied and shown to be a reliable model for evaluation of the effectiveness of antibacterials. The rabbit model described by Norden (Norden, 1970) is a simple and reproducible system for the study of osteomyelitis. A rat model for simulating intraabdominal sepsis, either with known organisms or with mixed fecal flora cultures, has been developed by Onderdonk et al. (1974).

15

A simple model for keratitis and experimental deratitis in guinea pigs is available (Davis et al., 1977), as is a simple method to obtain reproducible intraocular infections in rabbits (Maylath & Leopold, 1955).

20

Models are even available for sexually transmitted diseases. Corbeil et al. (1979) have described a mouse model for disseminated gonococcal infections, using a gonococcal strain (N24) isolated from a human genital tract. The most successful model for studying gonococcal urethritis is produced by the inoculation of chimpanzees with human urethral exudate (Arka & Balows, 1986). Johnson et al. (1982) have described a method for experimentally inducing syphilis in rabbits.

25

30

Models of fungal infections are also available, e.g., for infection with fungi other than dermatophytes, intravenous injection of clinical specimens or culture isolates is used for the conversion of dimorphic fungi to

35

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the tissue phase. Overwhelming numbers of organisms are usually used, since such infections develop slowly and frequently are not fatal. An example of a non-fatal subcutaneous fungal infection model is that achieved by accomplishing a local infection with *C. albicans* in cortisone-preconditioned animals. Such models may be employed in the detection of topical antifungal agents and combinations.

Preferably, in the above models, animals infected with resistant microorganisms would be treated in at least two ways before progressing to clinical trials. In the first procedure, the infected animals will be treated with the inhibitor(s) for a predetermined time before the antibiotic(s) treatment is started. In a second procedure, the infected animals are treated with the antibiotic(s)/inhibitor(s) combination at the same time. In yet another procedure, the first regimen could be modified where the infected animals would be treated pre-treated with the inhibitor(s) alone, then followed by treatment with the combination of the antibiotic(s)/inhibitor(s).

### EXAMPLE III

#### Methylation Inhibitors Increase Antibiotic Susceptibility

To show that compounds capable of directly or indirectly inhibiting methylation were able to increase the susceptibility of bacteria to MLS antibiotics, the inventors first used methods similar to the disk susceptibility test described in Example II.

In both of the following procedures, the protocol involved obtaining *S. aureus* lawn cultures in agar Petri dishes. Freeze-dried *S. aureus* cultures from the ATCC [Strain 27660 (inducible resistant strain); strain 6538



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(susceptible strain)] were reconstituted in 5 ml sterile broth media. Either Trypticase Soy Broth (TSB, Casein peptone 17g, soya peptone 3g, dipotassium phosphate 2.5g, NaCl 5g and dextrose 2.5g, Lot X1582, Adams Scientific Inc., West Warwick, RI) or Brain Heart Infusion Broth (BHIB, Casein peptone 15g, meat peptone/BHI solids 12g, dipotassium phosphate, Dextrose 2.0g, yeast extract 5.0g and NaCl 5.5g, Lot DM1082, Scott Laboratories Inc., West Warwick, RI) were used, at concentrations of 30 and 42g/l respectively.

The inoculated broths were incubated for 24h at 37°C, when they were streaked out on appropriate agar plates to assess purity of culture. Either of 40g/l BBL Trypticase Soy Agar (TSA, Pancreatic digest of casein 15g, papaeic digest of soybean meal 5g, NaCl, 5.0g and agar 15g, Lot K6DCXY, Becton and Dickenson, Cockeysville, MD) or Brain Heart Infusion Agar (15g/l Agar, Scott Scientific Inc. West Warwick, RI, Lot C-0281 and 42g/l BHIB) were used.

In the first procedure, two BHIA agar plates (Falcon Petri-dish 50 mm, Becton Dickenson, Lincoln Park, NY) were prepared, with an inhibitor mixed into one of the plates at the appropriate concentration. Both plates were inoculated with, e.g., a *Staphylococci* species resistant to erythromycin, using a sterile, aseptic wooden cotton swap containing about 0.2 ml of a  $10^6$  organism/ml solution. Two disks, each impregnated with erythromycin (15 mg/disc; Remel, Lenexa, Kansas), are placed on each agar plate. The plates were incubated for 17-24 hours then the clear zones were analyzed.

Although BHIA contains about 3% protein in contrast to the 2% protein in TSA, it is suspected that TSA contains higher concentrations of methionine and/or

- 84 -

growth factors that increase the robustness of *S. aureus* to erythromycin.

FIG. 1 shows the results from one such study in which erythromycin susceptibility for *Streptococcus aureus* 6538 (erythromycin susceptible) was examined in the presence of erythromycin in combination with ethionine, cycloleucine and trimethoprim. FIG. 2 shows similar results but using the erythromycin resistant strain *S. aureus* 27660. Ethionine, cycloleucine and trimethoprim all result in an increase in the size of the clear zone.

In a second evaluation procedure, 100mm Petri-dish agar plates (American Precision Plastic, North Geln, Co.) were prepared, as described in the first procedure, but this time with no inhibitor pre-mixed into the agar. Five discs containing 310  $\mu$ g ethionine/disc containing disc were placed on the *S. aureus* (resistant) incubated BHIA agar plate at zero time. At times 0, 1, 2, 3, and 5 hours, each disc was then removed and replaced with an erythromycin disc at the identical position that the inhibitor disc had previously occupied. Erythromycin controls were included at each point.

25

Increases in clear zones were observed after 18 hours incubation with inhibitor (ethionine)/erythromycin combination relative to erythromycin alone (FIG. 3). From these results it appears that pre-incubation with inhibitor for increased time periods increases the sensitivity of the resistant organisms to erythromycin. It is also contemplated that a broth susceptibility test will be suitable for analysing the effects of inhibitors.

35

Using the first procedure described above, various other inhibitors were also tested. At concentrations of

between 18 mM (for methotrexate) and 410 mM (for nicotinamide), all of the agents except SAH tested proved to increase the size of the clear zone (Table 8). The results are expressed in terms of relative effectiveness.

5

TABLE 8  
ERYTHROMYCIN SUSCEPTIBILITY FOR *S. AUREUS* 27660

Inhibitor	Concentration mM	Clear Zone mM	% Relative effectiveness <sup>3</sup>
Control	0	10 <sup>2</sup>	100
Methylthioadenosine	42 <sup>1</sup>	12.5	183
Nicotinamide	410	12.5	183
Na <sup>+</sup> Sulfadiazine	56	12.5	183
3-Deazaneplanocin A	31	12	167
2-Deoxyadenosine	183	14	233
DFMO	46	14.5	250
Ara-A	39 <sup>1</sup>	12.8	193
D,L-Buthionine-(S,R)-Sulfoximine	90	12	167
SJBA	33 <sup>1</sup>	12.8	193
Tubercidin	34 <sup>1</sup>	12.8	193
Methotrexate	18	14	233
S-Adenosylhomocysteine	35 <sup>1</sup>	10	100
2-Chlorodeoxyadenosine	35 <sup>1</sup>	12	167

<sup>1</sup> Contained 16% Dimethylformamide (DMF)

<sup>2</sup> Controls with and without DMF identical

<sup>3</sup>  $\frac{[D_{mod} - D_{disc}]}{[D_{Cont} - D_{Disc}]} \times 100$

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As shown in Table 8, with the great majority of the inhibitors, the bacterial lawn was observed to be substantially inhibited in comparison to the controls with no inhibitor. It is more than likely that SAH is not taken up by *S. aureus*. Walker & Duerre (1975) showed with studies in dogs that <sup>3</sup>H-labeled SAH is not taken up and excreted unchanged in urine. There exists a high likelihood that a similar effect occurred in the present study with SAH.

10

Importantly, the inhibitors shown in Table 8 are generally representative of the different groups of inhibitors identified by the inventors, i.e., compounds capable of inhibiting RNA methyltransferases; SAH hydrolase; SAM synthetase (via inhibiting glutathione synthetase); dihydrofolate reductase (DHFR); and also, agents that inhibit polyamine synthesis.

15

It should be noted here that the inventors currently contemplate that the use of other analytical methods will allow the above enhancement data to be refined. This is because the disc testing protocol, while clearly very useful, is not considered by the inventors to be the optimal protocol for a quantitative type of study. As such, a broth culture procedure, where the organism is grown in the presence of both erythromycin and the inhibitor in liquid medium, is proposed. The growth of bacteria is then assessed photometrically (Example II). This procedure is contemplated to allow a better evaluation of the potency of the inhibitors.

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As a reference point, it is important to note that erythromycin, as an example of an MLS antibiotic, is used clinically at an oral dose of 2000 mg/day (Reese & Betts, 1993). In vitro, in an agar disc susceptibility test, a MIC<sub>90</sub> (minimum inhibitory concentration for 90%

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- 87 -

inhibition) of about 4  $\mu\text{g/ml}$  for *S. aureus* is typically determined, while the MIC in broth culture for the same organism is typically between 0.12 and 0.5  $\mu\text{g/ml}$  (Weideman & Atkinson, 1991).

5

#### EXAMPLE IV

##### Inhibitors of Transmethylases

###### SAH and SAH Analogues

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SAH hydrolase (SAHH) occurs in many procaryotic organisms (Shimizu et al., 1984). It is believed that SAHH plays an important role in many cells in removing SAH, a product of transmethylation reactions involving SAM (Thong et al., 1985). SAH is an inhibitor of most SAM-dependent transmethylase reactions, consequently inhibition of SAHH gives rise to higher concentrations of SAH which reduces the activity of the transmethylases.

15

Yebra et al. (1991) showed that SAH (100  $\mu\text{M}$ ) and sinefungin (50  $\mu\text{M}$ ) inhibit RNA methyltransferase from *Streptomyces antibioticus* by 97 and 96% respectively. From these results, the inventors estimated that SAH dosage ranges similar to that of sinefungin could be used clinically. Due to the membrane transport characteristics of SAH it is anticipated that the most advantageous method of SAH delivery will be as a liposome or nano particle (Couvreur et al., 1991; Example XIII). Since the net effect of SAHH inhibition is to raise the SAH concentration which causes the inhibition of transmethylation reactions, it is expected that SAH will be used alone or in combination with other methylation inhibitors. Doses useful in therapy with antibiotics, will be from about 1 mg/kg/day to about 100 mg/kg body weight/day, and preferably, from about 5 to about 50 mg/kg body weight/day.

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- 88 -

Coward et al. (1974) reported that 2-fluoro-S-adenosylhomocysteine (2-FSAH) is an effective inhibitor of SAHH. Other structural analogues of SAH with modification in the amino acid, base or sugar portion of the molecule have been evaluated (Pugh & Bocharadt, 1982) in vitro for their abilities to inhibit the SAM-dependent transmethylation catalyzed by vaccinia virion mRNA(guanine-7)methyltransferase and mRNA(nucleoside-2'-)methyltransferase. S-Tubercidinylhomocysteine (STH) was found to be a particularly potent inhibitor of (nucleoside-2')methyltransferase.

From the candidate inhibitors of vaccinia virion mRNA(guanine-7)methyltransferase evaluated at 100  $\mu$ M, Pugh & Bocharadt (1982) showed that S-adenosyl-L-homocysteine sulfoxide (47% inhibition), S-adenosyl-L-homocysteine sulfone (40%), S-(N<sup>6</sup>-methyladenosyl)-L-homocysteine (85%), S-(3-deazaadenosyl)-L-homocysteine (84%), and S-aristeromycinyl-L-homocysteine (66%), sinefungin (100%) and A9145c (100%) were the most potent analogs.

Gillet et al. (1979) also described a number of SAH analogues mainly modified in the amino acid portion of the molecule, that are all effective competitive inhibitors of protein methyltransferase II from human erythrocytes. From these analogues, the inventors propose that 5'-S-(3-carboxyl-4-nitrophenyl)thioadenosine, S-adenosyl-L-homocysteine sulfoxide and 5'-S-(methyl)-5'-S-(butyl)thioadenosine would be particularly useful in connection with this invention.

The following doses are proposed for use in killing bacteria, and other microorganisms, in combination with

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antimicrobial agents: between about 1 to about 200, and preferably, between about 10 to about 100 mg/kg body weight/day for 2-FSAH and S-aristeromycinyl-L-homocysteine (STH); between about 1 to about 200, and preferably, between about 10 to about 100 mg/kg body weight/day for S-Adenosyl-L-homocysteine sulfoxide and S-Adenosyl-L-homocysteine sulfone; and between about 1 to about 200, and preferably, between about 5 to about 75 mg/kg body weight/day for S-(N<sup>6</sup>-methyladenosyl)-L-homocysteine and S-(3-deazaadenosyl)-L-homocysteine.

### Homocysteine

Homocysteine is mainly methylated by two different enzymes in the last step of the biosynthetic pathway in bacteria to form methionine, which is essential for the synthesis of SAM. The two enzymes are homocysteine methylase [EC 2.1.1.14; using N<sup>5</sup>-methyl tetrahydropteroylglutamate (N<sup>5</sup>>2) as methyl donor] and homocysteine methylase [EC 2.1.1.13; using vitamin B12 as methyl donor]. *E. coli*, as an example, contains both enzymes, whereas most organisms possess only one enzyme (Cohen & Saint-Girons, 1987). The inhibition of any of the biosynthetic reactions involved in the synthesis of homocysteine, is predicted by the inventors to inhibit transmethylation reactions since it increases the SAH/SAM ratio.

### S-N<sup>6</sup>-methyladenosylhomocysteine

S-N<sup>6</sup>-methyladenosylhomocysteine is nearly as potent an RNA methyltransferase inhibitor as SAH (Hoffman, 1978). Administration of N<sup>6</sup>-methyladenosine has been proposed as a general method for blocking *in vivo* RNA methylation in studies to determine the role of methylation in RNA processing and translational function.

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S-N<sup>6</sup>-methyladenosylhomocysteine may be synthesized in vitro from N<sup>6</sup>-methyladenosine and homocysteine in a reaction catalyzed by S-adenosylhomocysteine hydrolase.

5           S-N<sup>6</sup>-methyladenosylhomocysteine is commercially available from Sigma Chemical Company and may be used, in combination with an antimicrobial agent at doses of between about 1 to about 100 mg and preferably, between about 5 to about 50 mg/kg body weight/day.

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#### **Analogues of S-Aristeromycinyl-L-Homocysteine**

A series of base- and amino acid modified analogues of S-aristeromycinyl-L-homocysteine, a carbocyclic  
15 nucleoside, were synthesized and evaluated as inhibitors of SAM-dependent methyltransferases (Houston et al., 1985a). Houston et al. (1985a) observed that from the evaluated compounds S-aristeromycinyl-D-homocysteine and 3-deazaaristeromycinyl-D-homocysteine were particularly  
20 effective methylation inhibitors with K<sub>i</sub> of 10.4 and 20.5 μM, respectively, for phenylethanolamine N-methyltransferase.

These carbocyclic analogues are believed to be  
25 improved inhibitors in vivo as they will be more stable to metabolism than their ribosyl analogues (Houston et al., 1985a). Doses of between about 1 to about 100, and preferably, between about 5 to about 50 mg/kg body weight/day are proposed for these analogues in  
30 combination with antimicrobial agents such as MLS antibiotics.

#### **Polyinosinate**

35           Liau et al. (1973) have shown that polyinosinate inhibits transfer and ribosomal RNA (tRNA and rRNA)



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methylases. The mechanism of inhibition has been attributed to specific interactions between the single-stranded polyinosinate and the methylating enzymes. Methylation of individual bases were differentially affected by poly(I): inhibition of adenine methylases occurred to the greatest extent and guanine methylases to the least. Local administration of 12  $\mu$ g/mouse of poly(I) every 12 hours for 15 consecutive doses has been reported to inhibit multiplication of *Mycobacterium leprae* in the footpad of mice (Levy & Merrigan, 1977).

Polyinosinate with molecular mass ranging from 5000-200,000 is preferred for use in the present invention. From the data of Liau et al. (1973) and Levy & Merrigan (1977), the inventors contemplate that concentrations of poly(I) useful in therapy with agents such as MLS antibiotics, will be from about 0.1 mg/kg/day to about 30 mg/kg/day, and preferably, from about 0.1 to about 10 mg/kg/day. Poly(I) has been proposed for use in cancer chemotherapy and is thus considered suitable for human administration. Polyinosinate is commercially available from P-L Biochemicals (Milwaukee, Wis.).

Poly(I) was also found to be particularly effective in combination with adenine and adenosine (Liau et al., 1973). Extending the above schemes, it is thus also contemplated that adenine or adenosine could be combined with poly(I), with the adenine or adenosine being in a dose of from about 1 to about 700 mg/kg/day, and preferably, of from about 1 to about 500 mg/kg body weight/day.

#### S-Tubercidinylhomocysteine (STH)

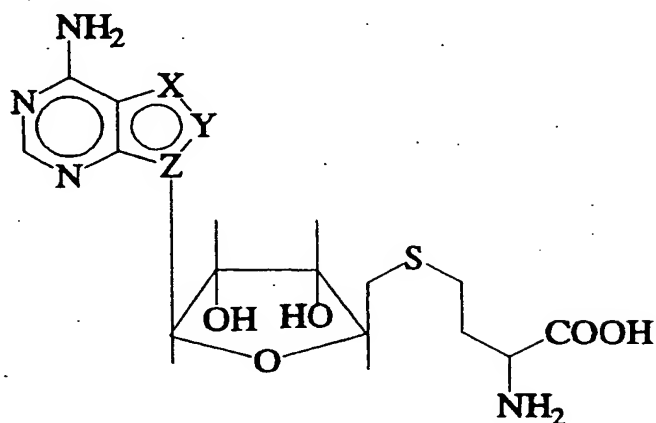
S-Tubercidinylhomocysteine (STH) has a stable ribose-purine bond and retains the inhibitory potency of

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SAH on tRNA methylases. The efficacy of STH parallels that of SAH. The synthesis of STH in particular is described in Coward et al. (1974) and its structure is depicted below:

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X = NH; Y = N; Z = C (SFH)  
X = CH; Y = CH; Z = N (STH)

20 STH has been used to study the methylation of mRNA in vivo. Inhibition of cytoplasmic methylation of poly(A)-containing mRNA poly(A)-RNA methylation has been observed using a level of inhibitor that still permitted cell growth (Kaehler et al., 1977). The use of STH as a  
25 methylation inhibitor offers several advantages since it is able to permeate cell membranes, and is not susceptible to the nucleosidase enzymes responsible for SAH metabolism in mammalian cells.

30 Chang & Coward (1976) also synthesized SAH analogues in which the 5'-thioether linkage is replaced by an oxygen or nitrogen isostere. These compounds were designed to be resistant to enzyme-catalyzed hydrolytic cleavage of the 5'-substituent. Although these compounds  
35 showed lower inhibition of catechol O-methyltransferases,

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they are contemplated for use as inhibitors of rRNA methylases.

STH is proposed for use in killing microorganisms in combination with antimicrobials at doses of between about 1 to about 100, and preferably, between about 5 to about 50 mg/kg body weight/day; with the analogues described above being useful in the range of about 1 to about 100, and preferably, about 70 to about 75 mg/kg body weight/day.

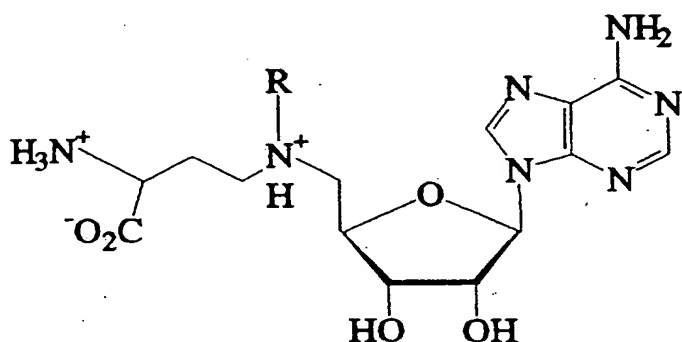
#### SAM Analogues

The synthetic approach to stable nitrogen alkyl SAM analogues, in which the sulfur atom is replaced by a nitrogen atom, has been described (Minnick & Kenyon, 1988). The procedures described have been used to prepare the methyl, ethyl, *n*-propyl, allyl, *n*-butyl, *n*-pentyl, *n*-octyl and 6-amino-1-hexyl nitrogen analogues of SAM. Testing one such nitrogen SAM analogue (in which R = Me in the structure below) with *E. coli* transfer RNA (uracyl-5)-methyl transferase has revealed that this compound functions as an inhibitor, but not as a methyl group donor (Minnick & Kenyon, 1988). The methyl analogue has been shown to have a  $K_i$  of 9  $\mu$ M for the *E. coli* methionine synthase.

It is expected that these derivatives will compete with all enzyme catalyzed reactions involving SAM. Such compounds, including the nitrogen analogues described below, are generally proposed for use in killing microorganisms in combination with antimicrobial agents, e.g., MLS antibiotics, at doses of between about 1 to about 100, and preferably, between about 5 to about 50 mg/kg body weight/day.

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Analogues of SAM in which the sulfur atom is replaced by a nitrogen atom have the advantage that they will not be susceptible to decomposition reactions peculiar to SAM. The structures of such analogues are shown:



R=Me

R=n-Bu

R=Et

R=n-Pe

R=n-Pr

R=n-Oc

R=CH<sub>2</sub>=CHCH<sub>2</sub>R=H<sub>2</sub>N(CH<sub>2</sub>)<sub>6</sub>

### Sulphonium SAM Derivatives

Zappia et al. (1969) described the preparation, purification, analysis of a variety of sulphonium derivatives of methionine. The methyl donor capacity of the inosine derivatives, and of decarboxylated S-adenosylmethionine, ranged between inactivity and effect equal to that of the biological methyl-donor SAM. In addition to SAH, these studies showed that S-inosyl-L-methionine, S-adenosyl-(5')-3-methylthio-propylamine, and 5'-(methylthio)adenosine were effective inhibitors.

The structures of active SAM sulphonium analogues are shown below. Such compounds, including the nitrogen analogues described below, are generally proposed for use

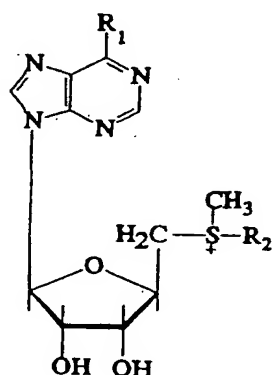
- 95 -

in killing microorganisms in combination with, e.g., MLS antibiotics, at doses of between about 1 to about 150, and preferably, between about 5 to about 75 mg/kg body weight/day.

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R <sub>1</sub>	R <sub>2</sub>
-NH <sub>2</sub>	-CH <sub>2</sub> CH <sub>2</sub> CH(NH <sub>2</sub> )COOH
-NH <sub>2</sub>	-CH <sub>2</sub> CH <sub>2</sub> CH(OH)COOH
-OH	-CH <sub>2</sub> CH <sub>2</sub> CH(OH)COOH
-OH	-CH <sub>2</sub> CH <sub>2</sub> CH(NH <sub>2</sub> )COOH
-NH <sub>2</sub>	-CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> NH <sub>2</sub>

20

### 5'-deoxy-5'-S-isobutyl-adenosine (SIBA)

5'-deoxy-5'-S-isobutyl-adenosine (SIBA) is known to be a competitive inhibitor of SAM and to strongly inhibit methylation in whole cells (Bona et al., 1976). SIBA inhibits the methylation of eukaryotic mRNA, especially that of the 5' cap, and it also affects various mRNA methylation to different extents (Jacquemont & Huppert, 1977).

SIBA also influences cell transformation by Rous sarcoma virus. Robert-Gero (1975) reported that adenosine homocysteine was less active as an inhibitor than its analogues and SIBA appeared to be the most

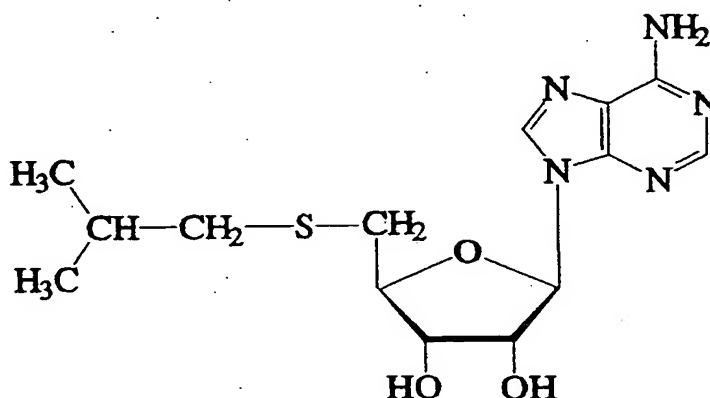
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potent among the latter at concentrations that were not toxic to normal cells.

SIBA has a structure as shown below, and position 2 is commercially available from Sigma Chemical Company.

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From established data, such as that from Jacquemont & Huppert (1977) showing that RNA methylation is inhibited ~65% by 1 mM SIBA, the inventors contemplate that therapeutically useful antibacterial concentrations of SIBA, when present with an antimicrobial agent, such as an MLS antibiotic, will be from about 1 to about 100 mg/kg body weight/day, and preferably, from about 10 to about 40 mg/kg body weight/day.

30

SIBA (0.033 M) was evaluated by the inventors and shown to enhance the relative activity of erythromycin against *S. aureus* 27660 by 193% (Table 8).

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### Sinefungin and A9145c

Sinefungin is an antifungal antibiotic isolated from cultures of *Streptomyces griseolus* (Vedel & Robert-Gero, 1981). Sinefungin very strongly inhibits the methylation of G in position 2 and 7, and that of A in position 1 and 6.

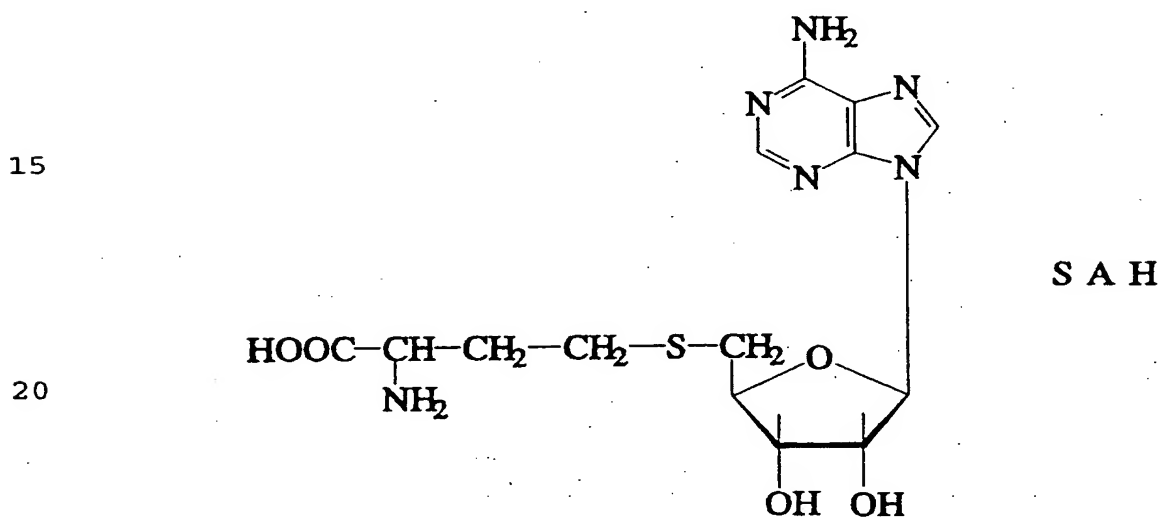
Sinefungin and a related metabolite, A9145C, were also found to be potent inhibitors of Newcastle disease virion mRNA- and vaccinia virion mRNA(guanine-7-)-methyl-transferase and vaccinia virion mRNA(nucleoside-2'-)-methyltransferase. Sinefungin and A9145C were tested as inhibitors of these methylases and both were found to be extremely active. At drug concentration of 10  $\mu$ M, where significant inhibition of virion plaque formation was observed, no cellular toxicity was detected. Although, at higher concentrations of sinefungin and A9145C, significant cellular toxicity was observed (Pugh et al., 1978), it has now been shown by Lemeteil et al. (1993) that sinefungin could be used in a rat model against *Cryptosporidium parvum* at a dosage of 10 mg/kg/day. Additionally, Avila et al. (1990) reported the optimum dosage using a mice model for cutaneous Leishmaniasis was 4 mg/kg/day, 50 times lower than the LD<sub>50</sub>.

Yebara et al. (1991) also showed that sinefungin and derivatives strongly inhibit RNA methyltransferases from *Streptomyces* (*S. antibioticus*, *S. incarnatus*, *S. griseolus*). Additionally it was shown that sinefungin and its analogues inhibit both RNA and DNA methyltransferases from these organism. *S. antibioticus* methyltransferase is inhibited at the 100  $\mu$ M level by A9145C (95%), cyclosinefungin (50%), SIBA 55%, ISOSIBA (52%) and 5'-S-methylthiomethyladenosine (65%). These

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results are in agreement with Li et al. (1985), who showed that sinefungin inhibits methylation of rRNA in *S.cerevisiae* and leads to a decrease in stable 18S rRNA. It is therefore expected that sinefungin and its analogues will function well with antibiotics and antimicrobials as claimed in the invention.

Sinefungin is commercially available from Sigma Chemical Company. The structure of sinefungin, in relation to that of SAH, is shown below.

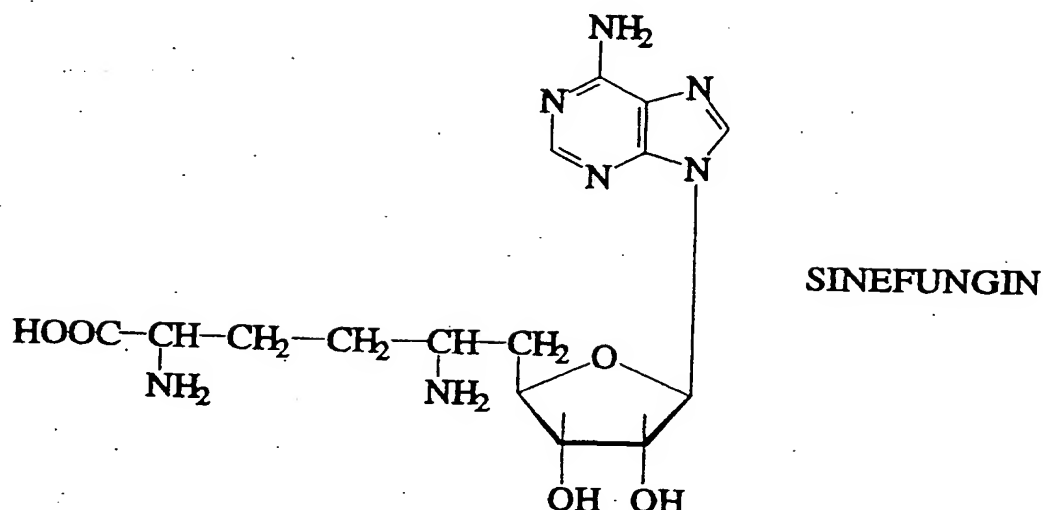


The synthesis of sinefungin has been reported by Barton et al., (1991) which allows modifications of the base portion of sinefungin to be prepared. Uracil analogues of sinefungin have also been prepared from uridine and L-aspartic acid (Barton et al., 1992), but are generally believed to have decreased inhibitory activity.

Based on the reports of Lemeteil et al. (1993) and Avila et al. (1990), it is contemplated that sinefungin concentrations of between about 0.1 and about 150 mg/kg



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body weight/day, and preferably, between about 1 to about 50 mg/kg body weight/day, may be used in combination with, e.g., MLS antibiotics, to kill bacteria.

## 5 Adenine Derivatives

Moore (1970) described the differential inhibition of tRNA methylases by adenine derivatives such as adenine, dimethylallyladenine, isopentenyladenosine, adenine phosphate, adenine sulfate, adenine-N<sup>1</sup>-oxide, 6-methoxyadenine, 6-dimethyladenine, 6-methyladenine and 6-mercaptadenine. It was found that all such compounds gave inhibition that would be acceptable for the purposes of the present invention, with the possible exceptions of 6-methoxyadenine and 6-dimethyladenine, and that dimethylallyladenine and isopentenyladenosine were particularly effective.

Therefore, adenine, adenine phosphate, adenine sulfate, adenine-N<sup>1</sup>-oxide, 6-methyladenine and 6-mercaptadenine may be used in combination with agents such as MLS antibiotics at doses of between about 10 to

- 100 -

about 200, and preferably, between about 25 to about 100 mg/kg body weight/day; with dimethylallyladenine and isopentenyladenosine having proposed doses of between about 1 to about 100, and preferably, between about 10 to  
5 about 75 mg/kg body weight/day.

### Nicotinamide

Swiatek et al. (1973) have shown that the inhibition  
10 of porcine liver tRNA methyltransferase activity by nicotinamide is due to an active methyltransferase enzyme that, in the presence of SAM and nicotinamide, produces 1-methylnicotinamide and SAH, the latter of which inhibits methyl transferase enzymes.

15

Nicotinamide (0.41 M) was evaluated by the inventors with the first procedure and shown to enhance the relative activity of erythromycin against *S. aureus* 27660 by 183% (Table 8). The sc. LD<sub>50</sub> of nicotinamide is  
20 1.68g/kg in rats (Windholz et al., 1983). It is therefore proposed that dosage of 1 mg/kg to 1.5 g/kg/day in combination with, e.g., MLS antibiotics, will kill bacteria and other microbes, the preferred dosage being  
100-1000 mg/kg/day.

25

### Methinin

Methinin is a natural occurring methylation inhibitor that is isolatable from rat and that inhibits a  
30 number of methyltransferases. Methinin is a low molecular weight compound (1400) that has an active amine group and appears to be of a peptide nature (Lyon et al., 1987). It inhibits DNA methylation, and has potential for use with antimicrobial agents in the present  
35 invention.

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**5'-Deoxy-5'-(methylthio)adenosine (MTA)**

MTA is an inhibitor of several SAM-dependent methylations (Law et al., 1992) and, in addition, is also an inhibitor of spermine and spermidine synthesis (Yamanaka et al., 1987). Wolford et al. (1984) injected MTA in mice ip. for 28 days. Single injections of as high as 75 mg/kg were administered. The inventors suggest that MTA at dosages of 1 to 200 mg/kg, and preferably, of 10 to 75mg/kg/day, in combination with, e.g., MLS antibiotics, will be effective in killing microorganisms. MTA is commercially available from Sigma Chemical Company.

**15 Xylosyladenine (9-B-D-xylofuranosyladenine)**

Garrett & Kredich (1981) showed that xylosyladenine (9-B-D-xylofuranosyladenine) caused marked inhibition of RNA methylation and showed that methyluridine, 2'-O-methylcytidine, 5-methyluridine and 5-methylcytidine formation in RNA was reduced. DNA methylation was not inhibited. From data such as this, it is proposed that xylosyladenine and other xylofuranosyl analogues of SAM or SAH, can be used with antimicrobial agents for killing microorganisms in doses of between about 0.1 to about 150 mg/kg body weight/day, and preferably, of between about 10 to about 40 mg/kg body weight/day.

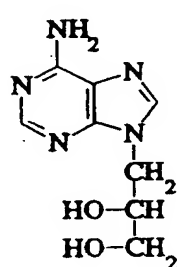
**EXAMPLE V****30 Inhibitors of SAH Hydrolase (SAHH)****Certain Adenosine Analogues**

S-adenosyl-L-homocysteine (SAH) hydrolase (SAHH) has been proposed as a target for antiviral agents (Wolfe & Borchardt, 1991). A good correlation exists between

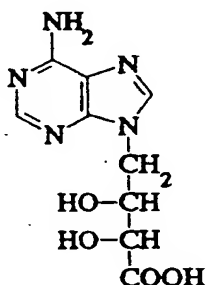
- 102 -

antiviral activity and SAHH inhibition. De Clercq & Cools (1989) observed a linear relationship between  $IC_{50}$  (concentration that inhibits replication by 50%) for a series of adenosine analogues and their  $\log K_i$  values for inhibition of murine L929 cell SAHH.

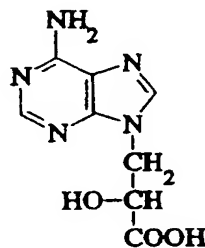
The following compounds have been described as first generation broad-spectrum antiviral agents: 9(S)-(2,3-dihydroxypropyl)adenine [(S)-DHPA], D-eritadine, (R,S)-3-adenine-9-yl-2-hydroxypropanoic acid [(R,S)-AHPA], adenosine (Ado) dialdehyde, 3-deazaadenosine ( $c^3$ -Ado), aristeromycin (Ari) and neplanocin A (NPA or NpcA) (De Clercq & Cools, 1985; 1989), the structures of which are shown below.



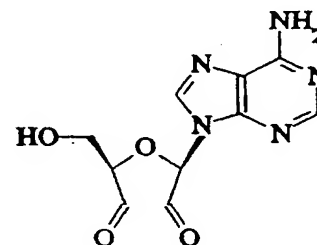
(S)-DHPA



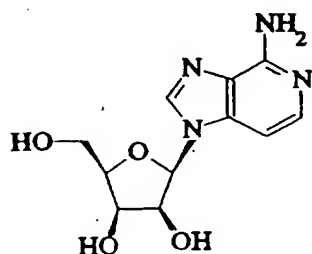
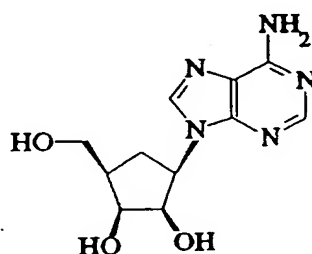
D-Eritadenine



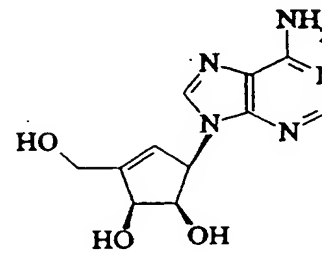
(RS)-AHPA



Ado dialdehyde

 $c^3$ -Ado

Ari



NpcA

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De Clercq & Cools (1989) also indicated that although these compounds are potent SAHH inhibitors, the cytotoxicity of these compounds may preclude clinical use as antiviral agents. However, since the  $ID_{50}/IC_{50}$  required in the antibacterial case is contemplated to be less stringent than that required for an antiviral agent, the inventors contemplate that such first generation compounds may be effectively used in the present invention at concentrations that are not cytotoxic. Additionally, these compounds could be used with inhibitors of adenosine kinase and adenosine deaminase (Wolfe & Bocharadt, 1991) to reduce toxicity.

The  $ID_{50}/IC_{50}$  ratio required for safe clinical use of the proposed inhibitor combinations with antimicrobial agents, such as MLS antibiotics, is assessed by determining the  $ID_{50}$  and  $IC_{50}$  experimentally using mice or rats. The  $ID_{50}$  and  $IC_{50}$  values are determined as described, e.g., by Cleeland & Squires (1991) and Klaassen (1990).

As shown by Wolfe and bocharadt (1991), SAH/SAM ratio ranges associated with therapeutic effectiveness ( $IC_{50}$ ) and cytotoxicity ( $ID_{50}$ ) can be determined, e.g., using HPLC. The concentrations of candidate inhibitors used clinically in combination with antibiotics will be determined using the  $IC_{50}$ ,  $ID_{50}$  and SAH/SAM ratios as determined above. Such concentrations are currently contemplated to lie in the general ranges stated below.

The activity spectrum of (D)-eritadine is similar to that of (S)-DPHA (Schanche et al., 1984). D-eritadine, L-eritadenine and L-threo-eritadenine inactivate SAH in hepatocytes. Proposed doses for use in microbial killing, when combined with, e.g., MLS antibiotics, of between about 1 to about 100 mg/kg body weight/day), and

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preferably of between about 10 to about 75 mg/kg body weight/day, are proposed for the eritadenine derivatives.

De Clercq & Cools (1985) reported the inhibition of  
5 SAH hydrolase by the following compounds at the stated concentrations: (S)-DPHA ( $K_i = 1.4 \mu\text{M}$ ); (RS)-AHPA ( $K_i = 0.073 \mu\text{M}$ ), carbocyclic 3-deazaadenosine ( $K_i = 0.013 \mu\text{M}$ ) and NPA ( $K_i = 0.002 \mu\text{M}$ ). Kitaoka et al. (1986) also showed inhibition and Backlund et al. (1986)  
10 showed that N- and O-methylation of mRNA was inhibited between 31-83% by  $100 \mu\text{M}$  3-deazaadenosine.

Since De Clercq & Cools (1985) demonstrated a correlation between the SAH inhibition potency and their  
15 antiviral potencies, it is expected that similar effects will be seen on the inhibition of rRNA methylation by these compounds. Also, as (S)DHPA, (R,S)-AHPA isobutylester, carbocyclic-3-deazaadenosine (C- $c^3\text{Ado}$ ) and NPA reduce vesicular stomatitis virus-induced  
20 cytopathogenicity by 50% in primary rabbit kidney cells, with minimum inhibitor concentration of 20, 2, 0.2,  $0.018 \mu\text{g/ml}$ , respectively, it is further proposed that such compounds will operate as methylation inhibitors at similar ratios.

25

Kitaoka et al. (1986) studied several nucleoside analogues known to have broad spectrum antiviral activity, e.g., ribavirin, vidarabine (Ara A), pyraazofurin, tubercidin, carbodine, [(S)-DHPA], (C- $c^3\text{Ado}$ ), [(RS)-AHPA] isobutyl ester and neplanocin A.  
30 These compared for their potency and selectivity as inhibitors of human retrovirus replication *in vitro*. These adenosine analogues were proposed to also owe their antiviral activity to the inhibition of SAH hydrolase.  
35 The studies of Kitaoka et al. (1986), reported effective inhibitory concentrations of (S)-DPHA, C- $c^3\text{Ado}$ , (RS)-AHPA

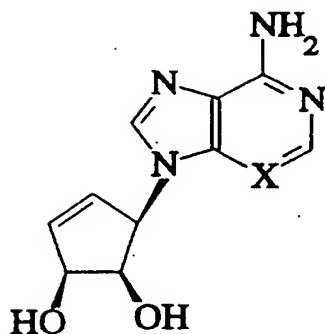
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isobutyl ester and NPA are about 60, 1.4, 1.2 and 0.2  $\mu\text{g/ml}$ , respectively. In vitro cytotoxicity indicated an activity index of >3, 70. 80 and >20 respectively for (S)-DPHA), C-C<sup>3</sup>Ado, (RS)-AHPA isobutyl ester and NPA, suggesting a potential for *in vivo* use.

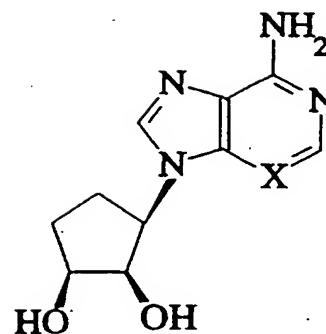
The inventors have translated the above information into proposed doses for use in killing microorganisms, when combined with, e.g., MLS antibiotics, of between about 0.1 to about 100 mg/kg body weight/day, and preferably, of between about 0.1 to about 30 mg/kg/day for (S)-DPHA, C-C<sup>3</sup>Ado, (RS)-AHPA, Ari and NPA.

Wolfe and Borchardt (1991) also described second generation SAHH hydrolase inhibitors that have retained antiviral activity and considerably lower cytotoxicity than their parent first generation inhibitors. These compounds include: dihydroxycyclopentenyladenine (DHCEA) and dihydroxycyclopentenyl-3-deazaadenine (c<sup>3</sup>-DHCEA); dihydroxycyclopentanyladenine (DHCaA) and dihydroxycyclopentanyl-3-deazaadenine (c<sup>3</sup>-DHCaA); 3-deazaneplanocin A (c<sup>3</sup>-NpcA) and 3-deazaaristeromycin (c<sup>3</sup>-Ari), as shown below.

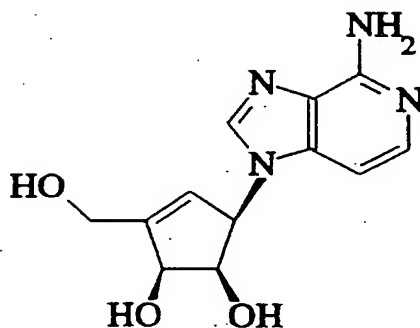
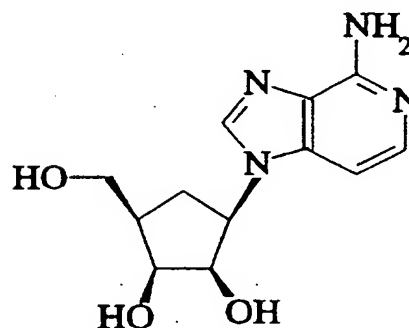
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DHCaA: X = N  
 c<sup>3</sup>-DHCaA: X = CH



DHCaA: X = N  
 c<sup>3</sup>-DHCaA: X = CH

c<sup>3</sup>-NpcA

Ari

The increased therapeutic effectiveness of the second generation inhibitors (ratio of ID<sub>50</sub>/IC<sub>50</sub>) is apparently due to the elimination of adenosine kinase and adenosine deaminase activity of these generation

5 compounds. It is contemplated that the second generation inhibitors will function successfully in clinical combinations with antimicrobial agents, as exemplified by MLS antibiotics. The safe clinical dose for the second generation inhibitors, when used in combination with,



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e.g., MLS antibiotics, will be higher than for the first generation inhibitors.

Using published information, such as that in De Clerq et al. (1989), the following doses are contemplated for using the second generation compounds in combination with antibiotics to achieve microbial killing: between about 0.1 and about 100; preferably, between about 0.1 and about 50; and most preferably, between about 0.1 and about 10 mg/kg body weight/day for DHCeA, c<sup>3</sup>-DHCeA, DHCaA, c<sup>3</sup>-DHCaA, A(c<sup>3</sup>-NpcA) and c<sup>3</sup>-Ari.

2'-deoxy-2'-chloroadenosine (CLA) was shown to be an inhibitor of hamster and human liver SAHH (Kim et al., 1985). Buckle et al. (1981) evaluated CLA at dosages of 300 µMol/kg (90 mg/kg) in mice. Based on these observations, it is proposed that CLA in combination with, e.g., MLS antibiotics, will provide enhanced killing at dosages between 1 and 150 mg/kg/day, and preferably, at between 10-90 mg/kg/day.

Methylthioadenosine (MTA, 0.042 M) and 2-Chlorodeoxyadenosine (0.035 M) were evaluated by the inventors with the first procedure and shown to enhance the relative activity of erythromycin against *S. aureus* 27660 by 183% and 163% respectively (Table 8). MTA is thus also proposed for clinical use with, e.g., MLS antibiotics, at dosages of between 1 and 150 mg/kg/day.

6-(γ,γ-Dimethylallylamino)purine riboside 1-200. 10-100 6-(γ,γ-Dimethylallylamino)purine riboside is commercially available from Sigma Chemical Company.

6-(Isopentenyl)adenosine 1-200. 10-100 6-(Isopentenyl)adenosine is commercially available from Sigma Chemical Company.

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6-Dimethylaminopurine riboside 1-200. 10-100

6-Dimethylaminopurine riboside is commercially available from Sigma Chemical Company.

## 5 Neplanocin A (NPA)

Neplanocin A (NPA) is a cyclopentenyl analogue of adenosine that is a potent inhibitor of SAH hydrolase. NPA has been shown to inhibit vaccinia virus  
10 multiplication in mouse L929 cells. Concentrations of NPA as low as 0.5 and 1  $\mu$ M in the culture medium have been reported to produce 84 and 95% inhibition of plaque formation, respectively, while exhibiting little toxicity  
15 to the host cells (Borchardt et al., 1984). This finding suggests that the antiviral action of this compound is related to an inhibition of SAM-dependent methylation reactions that are essential to the production of new viral particles (e.g. viral messenger RNA).

20 Yaginuma and colleagues reported that NPA possesses significant antitumor activity *in vivo* (Yaginuma et al., 1979; 1981; Tsujino et al., 1979). This indicates that NPA is safe for *in vivo* use.

25 Glazer & Knode (1984) also studied the activity of NPA against human colon carcinoma *in vitro*. They determined NPA to be an inhibitor of RNA methylation, but not of RNA synthesis (transcription). These properties confer to NPA unique pharmacological characteristics not  
30 possessed by other adenosine analogues studied at that time. Consequently, NPA should be a valuable tool for further chemotherapeutic studies.

NPA is available from the Toyo Jozo Co., Ltd., Japan  
35 and it may be synthesized according to the methods of Lim & Marquez (1983). From the studies showing that NPA

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inhibited malarial growth with  $ED_{50}$  of  $3\mu M$  (Whaun et. al., 1986), and because NPA does not cause cellular toxicity ((Yaginuma et al., 1979; 1981; Tsujino et al., 1979), it is contemplated that useful concentrations of NPA for use in killing microorganisms (in combination with antimicrobial agents) will be from about 1 to about 50 mg/kg body weight/day, and preferably, from about 1 to about 20 mg/kg body weight/day.

The inventors make this proposal despite certain prior teachings concerning NPA. For example, Fischer et al. (1987) studied the effect of Neplanocin A on sixteen bacteria cultured on agar plates, and only two were determined to be sensitive. None of the sixteen showed any growth sensitivity in broth culture to concentrations as high as 4 mM (Fischer et al., 1987). The authors concluded that SAH as a target for antimicrobial agent development seemed not likely to be a productive approach (Fischer et al., 1987).

In marked contrast to the teachings represented by Fischer et al. (1987), the present inventors have unexpectedly found that several SAH inhibitors very similar to Neplanocin A were effective in enhancing the action of MLS antibiotics. In particular, Ara-A, 3-Deazaneplanocin A, methylthioadenosine and 2'-deoxyadenosine, used in combination with erythromycin, enhanced the killing of resistant *S. aureus* 27660 bacteria (Table 8).

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**3-deazaneplanocin A ( $c^3$ -NPA)**

Tseng et al. (1989) described the synthesis of the neplanocin A analogue 3-deazaneplanocin A (also obtainable from Dr. V.E. Marguez; National Institutes of Health, Bethesda, Maryland). This derivative is neither phosphorylated nor converted to the S-nucleosidylhomocysteine analogue and is therefore significantly less cytotoxic than neplanocin A. 3-deazaneplanocin A is thus contemplated to be one of the preferred compounds for use in combination with one or more MLS antibiotics or other antimicrobial agents.

Glazer et al. (1986) showed that NPA (10  $\mu$ M),  $c^3$ -NPA (10  $\mu$ M) and  $c^3$ -Ari (100  $\mu$ M) inhibit the methylation of 28S rRNA, 18S rRNA and tRNA. Additionally it was shown that NPA,  $c^3$ -NPA and  $c^3$ -Ari showed cytotoxicity of HT-29 cells above 1, 100 and 250  $\mu$ M, respectively. It is therefore expected that  $c^3$ -NPA and  $c^3$ -Ari could be used at dosages 10-100 times higher than that of NPA, i.e., used (in combination with antibiotics) at doses of from about 0.1 to about 1,000 mg/kg body weight/day, and preferably, from about 0.1 to about 200 mg/kg body weight/day.

As a practical matter,  $c^3$ -NPA (0.031 M; from Dr. V.E. Marguez) was evaluated by the inventors using the first disc susceptibility test, when it was shown to enhance the activity of erythromycin against *S. aureus* by 167% (Table 8).

**Other Neplanocin A Analogues**

Shuto et al. (1992) synthesized a number of 6'-modified Neplanocin A derivatives, which were evaluated for their SAHH and antiviral activities. Each of the

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compounds described were particularly active against a number of viruses. Additionally, they showed increased selectivity with 6'-C-methylneplanocin A surpassing neplanocin A both in antiviral potency and selectivity.

5

6'-C-methylneplanocin A, virtually resistant to adenosine deaminase and a good inhibitor of murine L929 SAHH, is expected to function extremely well in combination with MLS antibiotics. It is contemplated that this agent may be used in combination with, e.g., MLS antibiotics, at doses of from about 1 to about 200 mg/kg body weight/day, and preferably, from about 1 to about 100 mg/kg body weight/day.

10

#### 15 Ara-A and Arabinoside Compounds

2'-Deoxyadenosine and 9-B-arabinofuranosyladenine (Ara-A) are suicide inactivators of SAH hydrolase (Hershfield, 1979; Helland & Ueland, 1982). Ara-A appears to be twice as active as 2'-deoxyadenosine. Ara-A is approved by the FDA for use as an ophthalmic ointment for herpes simplex virus infections and also as a systemic treatment for herpes simplex encephalitis at a dosage of 15 mg/kg/day for 10 days (Berkow et al., 1992). This suggested to the inventors that Ara-A could be used therapeutically.

20

25

Ara A (0.039 M) and 2'-deoxyadenosine (0.183 M) were evaluated by the inventors using the first disc susceptibility test, and shown to enhance the relative erythromycin activity against the resistant *S. aureus* 27660 by 193 and 233% respectively (Table 8). The proposed dosages for Ara-A and 2'-deoxyadenosine clinical use range between about 1-300 mg/kg/day, and preferably, between about 1-100 mg/kg/day. LD<sub>50</sub> of Ara-A in mice is

30

35

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4,677 mg/kg (i.p) and 7,950 mg/kg orally (Windholz et al., 1983).

Although Sacks et al. (1982) have suggested that the  
5 inactivation of SAH hydrolase could mediate toxic  
manifestations of ara-A therapy in certain group of  
patients, used at the appropriate therapeutic levels,  
Ara-A is contemplated to be an effective agent for  
combination with antibiotics. Both Ara-A and  
10 2-Deoxyadenosine are commercially available from Sigma  
Chemical Company (St. Louis, MO, USA) and Parke-Davis &  
Co. (Detroit).

15 **Aristeromycin (9- $\beta$ -{2,3-dihydroxy-4-(hydroxymethyl-  
cyclopentyl}adenine) and Analogues**

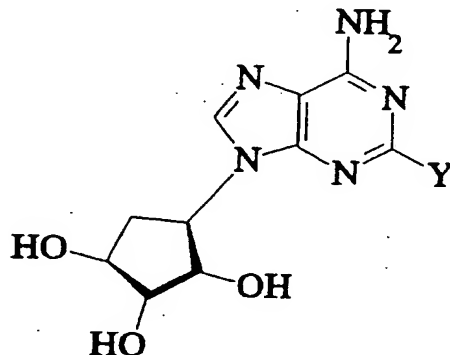
Houston et al. (1985b) synthesized a series of  
purine (adenine, N<sup>6</sup>-methyladenine, 8-azaadenine, 3-  
deazaadenine) carbocyclic nucleosides, nucleoside-2',3'-  
20 dialdehydes and nucleoside-2',3'-diols as potential  
inhibitors of SAHH and vaccinia virus replication. They  
observed a good correlation between a compound's  
inhibitory effect on SAHH and its antiviral effects,  
suggesting that viral inhibition is caused by the  
25 inhibition of a critical methylation step. Similar  
antimicrobial activity is expected from these compounds.

The most effective inhibitors of bovine liver SAHH  
from the synthesized series were evaluated at 0.2 mM  
30 Aristeromycin (99% inhibition), N<sup>6</sup>-methyларisteromycin  
(22%), 8-azaaristeromycin (46%) and 3-deazaaristeromycin  
(97%). The inhibition of the corresponding dialdehyde  
derivatives at 0.2 mM ranged from 32 to 100%, while the  
corresponding diol derivatives showed between 82 to 98%  
35 inhibition only at the 2 mM level. The % inhibition of  
vaccinia plaque formation by the compounds above at the

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10  $\mu$ M level followed to a large extent the ability to  
inhibit SAHH. Similarly, (Keller & Borchardt, 1987)  
showed that the dialdehyde derivative produces an  
increase in intracellular levels of SAH and subsequent  
5 inhibition of SAM-dependent methylations.

( $\pm$ )-5-Noraristeromycin and its 2,6-diamino-analogue  
were synthesized (Patil & Schneller, 1992; structures  
shown below) and shown to be inhibitors of SAHH. In  
10 antiviral evaluations it was observed that ( $\pm$ )-5-  
Noraristeromycin was 10 to 20 times less cytotoxic in  
cell culture. It is therefore expected that both these  
compounds will function well in combination with MLS  
antibiotics.



Y = H or NH<sub>2</sub>

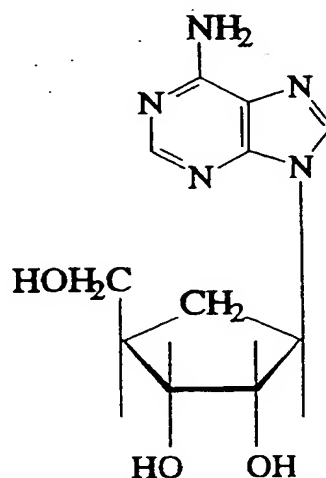
For aristeromycin and derivatives, the following  
doses are proposed for use in killing microorganisms in  
30 combination with antibiotics: between about 1 to about  
200, and preferably, between about 10 to about 100 mg/kg  
body weight/day for aristeromycin, 3-deazaaristeromycin,  
( $\pm$ )-5-Noraristeromycin and its 2,6-diamino-analogue;  
between about 1 to about 100, and preferably, between  
35 about 5 to about 50 mg/kg body weight/day for N<sup>6</sup>-  
methyларisteromycin; and between about 5 to about 200,

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and preferably, between about 10 to about 100 mg/kg body weight/day for 8-azaaristeromycin.

One of the cellular reactions affected by aristeromycin is transmethylation. Because of the sensitivity of the transmethylases to the S-aristeromycinyI analog of SAH, it is likely that the eukaryotic mRNAs or viral mRNA methyltransferases will be inhibited. Due to its cytotoxicity to mammalian cells, many derivatives have been synthesized to overcome toxicity. These include 2'-deoxy-, 3'-deoxy-, 3'-amino-3'-deoxy-, 3'-amino-3'-deoxyarabinofuranosyl, 6'-hydroxy, 6'-mercapto, 8'-bromo, 8-hydroxyaristeromycin, aristeromycin-3'-cyclic phosphate and aristeromycin-6'-cyclic phosphate. The appropriate doses for any given aristeromycin derivative will lie in the ranges described above, closest to the structural analogue which the derivative most closely resembles. Optimization of the dose will be routine given these guidelines.

The structure of aristeromycin is shown below, it is commercially available from Sigma Chemical Company.



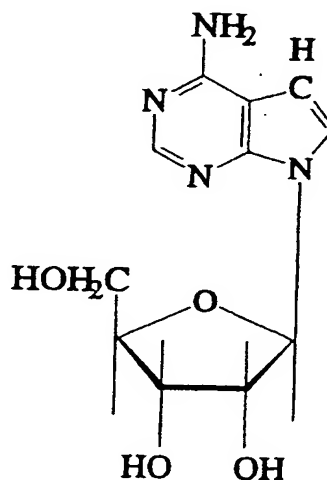


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**Tubercidin (7-deazaadenosine)**

Enzymes that methylate tRNA are known to have qualitative and quantitative differences among different species. Inhibitors of these enzymes with three different specificities have been identified, namely some restricted bacterial enzymes, some to mammalian enzymes, and others with dual potencies (Wainfan & Borek, 1967). Adenine, adenosine, isopentenyladenosine and tubercidin are inhibitory to tRNA methylases isolated from *E. coli* and thus inhibit methylation.

As tubercidin inhibits SAHH, the SAH/SAM ratio increases and inhibits methylation. This is one instance of a compound that exerts an effect at various points in the cell. Tubercidin and isopentenyladenosine are commercially available from Sigma Chemical Company. Tubercidin has the following structure:



Tubercidin also inhibits rRNA processing in addition to methylation of tRNA and protein and nucleic acid synthesis. The substitution of tubercidin for adenosine in SAH yields a potent inhibitor of tRNA methylase,

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Catechol-O-methyltransferase, indole ethylamine  
N-methyltransferase, methylation of tRNA in  
phytohemagglutinin-stimulated lymphocytes and polyamine  
biosynthesis.

5

Tubercidin (0.034 M) was evaluated by the inventors  
with the first procedure and shown to enhance the  
relative activity of erythromycin against *S. aureus* by  
193% (Table 8). The LD<sub>50</sub> of tubercidin iv. in mice is 45  
10 mg/kg (Windholz et al., 1983). Using this data, the  
inventors propose the following tubercidin dosage range  
for use with, e.g., MLS antibiotics, in killing bacteria:  
between about 0.1 to about 40, and preferably, between  
about 1 to about 20 mg/kg body weight/day.

15

#### EXAMPLE VI

##### Inhibitors of SAM Synthetase (SAMS)

#### 20 Cycloleucine

Cycloleucine (1-aminocyclopentane-1-carboxylic acid)  
is an inhibitor of SAM synthetase (SAMS, or methionine-  
adenosyltransferase). Finkel & Groner (1983) reported  
25 that cycloleucine, at 0.5 mg/ml, causes more than a 30%  
decrease in internal m<sup>6</sup>A of late SV40 mRNA, with only a  
minor effect on the dimethyladenosine of the 5' cap  
structure. After treatment with 2 and 5 mg/ml  
cycloleucine, internal m<sup>6</sup>As were reduced by 10- and  
30 100-fold, respectively.

Kroes et al. (1984) have shown that nitrous oxide  
and cycloleucine have synergistic growth inhibiting  
effects in experimental rat leukemia (BNML). These  
35 results indicated that a reduction of SAM synthesis by  
cycloleucine can increase the disturbance of folate

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metabolism that is caused by nitrous oxide, with a potentiation of the effects on leukemic growth.

Cycloleucine at a concentration of 0.1 M, pre-mixed  
5 with Brain Heart Infusion Agar, showed improved killing  
(240% increase over control) of *S. aureus* resistant to  
erythromycin (FIG. 2). From the above inhibitory range  
of 0.5 to 5 mg/ml and the LD<sub>50</sub> of 309 mg/kg in mice  
(Windholz et al., 1983), it is contemplated that  
10 therapeutic cycloleucine doses of between about 1 and  
about 250 mg/kg body weight/day, and preferably, of  
between about 10 and about 50 mg/kg body weight/day, will  
be of use in combination with antibiotics to treat  
infections. Cycloleucine is commercially available from  
15 Sigma Chemical Company.

#### Methionine Analogues

Sufrin et al. (1979) reported that mono-, bi- and  
20 tri-cyclic amino acid structural and conformational  
analogues of L-methionine functioned as inhibitors of the  
enzymatic synthesis of S-adenosyl-L-methionine. These  
synthetic amino acids are structurally and  
conformationally related to cycloleucine (described  
25 above). Compounds that function as effective inhibitors  
include cyclopentaneglycine, the (1R,2R,4S) isomer of  
2-aminonorbornane-2-carboxylic acid, the (1R,2R,4S)  
isomer of 2-amino-5,6-exo-trimethylenenorbornane-2-  
carboxylic acid, 3-aminobicyclo[3.2.0]heptane-3-  
30 carboxylic acid and, most effective, (+)-2-  
aminobicyclo[2.1.1]hexane-2-carboxylic acid.

The concentration for 50% inhibition of these  
compounds are similar to that of cycloleucine. It is  
35 therefore expected that similar concentration as those

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contemplated for cycloleucine will be therapeutically active in combination with, e.g., MLS antibiotics.

Lombardini & Sufrin (1983) described the  
5 chemotherapeutic potential of methionine analogue  
inhibitors of tumor-derived methionine  
adenosyltransferases. In addition to cycloleucine,  
1-aminocyclobutanecarboxylic acid,  
1-aminocyclohexanecarboxylic acid, L-2-amino-4-hexanoic  
10 acid, (Z)-L-2-amino-5-chloro-trans-4-hexanoic acid, L-  
ethionine, and seleno-DL-ethionine were found to be  
effective inhibitors, with  
(+)-2-aminobicyclo[2.1.1]hexane-2-carboxylic acid again  
being found to be the most effective. Since the  $I_{50}$  of  
15 these compound are similar to that of cycloleucine,  
similar therapeutic performance is expected.

Kramer et al. (1987) described the relative effects  
of SAM depletion on nucleic acid methylation and  
20 polyamine biosynthesis. It was shown that the treatment  
of cultured L1210 cells with 1 mM  
L-2-amino-4-methoxy-cis-but-3-enoic acid (L-cis-AMB), a  
methionine analogue inhibitor of SAM synthetase, produced  
a rapid and near-total depletion of SAM by 4 hours.  
25 Kramer et al. (1988) also reported that L-cis-AMB, the  
most potent methionine-analogue inhibitor of SAM  
synthetase at that time, is capable of rapidly depleting  
SAM pools in intact cells without affecting precursor  
incorporation into macromolecules.

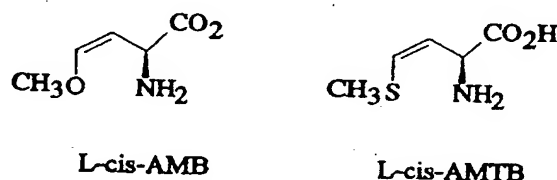
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Growth inhibition in L1210 cell culture cells by  
methionine analogue inhibitors of SAM biosynthesis in the  
absence of polyamine depletion has been reported by  
Porter et al. (1984). In addition to cycloleucine  
35 ( $IC_{50}$ =5 mM) and (+)-2-aminobicyclo[2.1.1]hexane-2-  
carboxylic acid ( $IC_{50}$  = 5 mM), this group identified

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selenomethionine ( $IC_{50} = 0.13$  mM) and L-cis-AMB ( $IC_{50} = 0.4$  mM) as effective inhibitors, with L-cis-AMB being more potent. The  $IC_{50}$  of L-cis-AMB is about 10 fold lower than that of cycloleucine: it is therefore  
5 expected that it will operate therapeutically at lower concentration in combination with, e.g., MLS antibiotics.

Sufrin et al. (1993) later described the activity of L-2-amino-4-methylthio-cis-but-3-enoic acid (L-cis-AMTB)  
10 as an inhibitor of SAM-synthetase. L-cis-AMTB was slightly less inhibitory than L-cis-AMB, but is nonetheless effective. The structures of L-cis-AMB and L-cis-AMTB are shown below:



20 For the methionine analogues, it is contemplated that concentrations generally around 1-150 mg/kg body weight/day may be used in combination with antimicrobial agents to kill bacteria and other microbes. More  
25 specifically, for (+)-2-aminobicyclo[2.1.1]hexane-2-carboxylic acid, a preferred therapeutic dose of between about 10 and about 50 mg/kg body weight/day is proposed; and for L-cis-AMB, a therapeutic dose of between about 1 and about 100 mg/kg body weight/day, and preferably, of  
30 between about 5 and about 50 mg/kg body weight/day is contemplated.

#### ATP Analogues

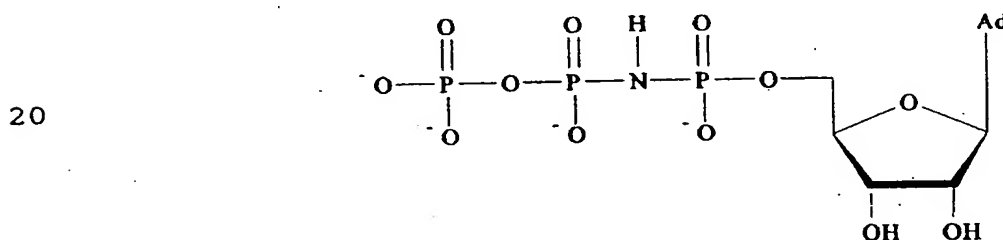
35 Ma et al. (1990) described a family of ATP analogues that are either mono- or di-substituted with imido and

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methylene bridges in the polyphosphate chain: adenosine 5'-( $\alpha,\beta:\beta,\gamma$ -diimidotriphosphate) (AMPNPNP), adenosine 5'-( $\alpha,\beta:\alpha\beta'$ -diimidotriphosphate) (AMP(NP)<sub>2</sub>), and adenosine 5'-( $\alpha,\beta:\beta,\gamma$ -dimethylenetriphosphate) (AMPCPCP).

5 These were investigated as substrates and inhibitors of SAM synthetase. AMPNPP was found to be both a substrate for SAM synthetase and a potent inhibitor, with an inhibition constant 60 fold less than the  $K_m$  of ATP. AMPNPP was about 1000-fold more effective as an inhibitor  
10 than AMPCPP.

The synthesis of AMPCPP and the other analogues is described in Ma et al. (1990), the structure of which is depicted below. Since the  $K_i$  value for AMPCPP is similar  
15 to that of cycloleucine, equivalent therapeutic potency is expected.



25

#### Further Inhibitors

Numerous inhibitors of SAM synthetase have been synthesized in the laboratory of Kappler. Their  
30 synthesis and biological activity have been reported in a number of papers (Kappler et al., 1986a; 1986b; 1987; 1988; Lim et al., 1986; Vrudhula et al., 1987; 1989).

Vrudhula et al. (1989) reported the synthesis of  
35 nonselective inhibitors of various isozymes of rat SAM synthetase. Four types of covalent methionine-ATP

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adducts were described to be potent inhibitors of the M-1 and M-2 isozymes. The most effective inhibitor is the 5'R epimer of a CH<sub>3</sub>-C5' adduct of L-methionine and  $\beta,\gamma$ -imido-ATP. This compound,

5 5' (R) - (C) - [(L-homocysteine-S-yl)methyl]adenosine 5'-( $\beta,\gamma$ -imidotriphosphate), is also described by Kappler et al. (1987).

### Inhibitors of Glutathione Synthetase

10

Corrales et al. (1991) showed that there is a close correlation between the hepatic levels of glutathione and SAM synthetase activity. These authors suggested that under normal conditions, oxidation of cysteine groups of SAM synthetase may be protected by the intra-cellular levels of glutathione. The SAM-synthetase activity of animals treated with buthionine sulfoximine was shown to be only about 40% of that of untreated animals.

20

DL-buthionine-(S,R)-sulfoximine or L-buthionine(S,R)-sulfoximine used in most studies contains 4 or 2 isomers, respectively (Campbell et al., 1991). These authors showed that the different isomers were effective in reducing GSH levels in mice liver, kidney and pancreas at the concentrations of 0.2 and 0.4 mmol/kg respectively with L-buthionine-(S)-sulfoximine and L-buthionine-(S,R)-sulfoximine. L-buthionine-(R)-sulfoximine did not show any significant depletion of GSH at 0.2 mg/kg in liver or pancreas, but modest depletion in the kidney.

30

Lasierra et al. (1989) determined that buthionine sulfoximine [4.5 mmol/kg (1g/kg) and diethyl maleate (3.2 mmol/kg) (0.55 g/kg)] ip. depleted liver and aortic glutathione in rabbits. Muzutani et al. (1994) suggested

35

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the use of buthionine sulfoximine could have application in tumor therapy.

As inhibitors of glutathione synthetase decrease the activity of SAM-synthetase, the present inventors thus propose the use of any glutathione synthetase inhibitors in combination with antibiotics. One such example is the buthionine sulfoximine mentioned above. Buthionine sulfoximine has the structure:

10  $\text{CH}_3(\text{CH}_2)_2\text{S}(\text{O})(=\text{NH})\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$ , and is commercially available from Sigma Chemical Company.

Based on the *in vivo* data presented by Corrales et al. (1991), and on the fact that the GSH levels can be depleted in a dosage dependent fashion (Drew et al., 1984), the inventors contemplate that suitable therapeutic doses of buthionine sulfoximine will be between about 1 and about 1000 mg/kg body weight/day, and preferably, of between about 1 and about 100 mg/kg body weight/day. The inventors have shown that when L-buthionine(S,R)-sulfoximine (0.09 M) was evaluated in a disc susceptibility assay it enhanced the relative activity of erythromycin against *S. aureus* by 167% (Table 8).

25

Many other examples of glutathione synthetase inhibitors have been described, any one or more of which may be used in connection with the present invention. Kato et al. (1987) showed sequence homology of over 40 amino acids between glutathione synthetase of *E. coli* and dihydrofolate reductase from *E. coli* and mammalian sources. It was shown that *E. coli* glutathione synthetase was potently inhibited by 7,8-dihydrofolate, methotrexate and trimethoprim.  $\alpha$ -aminomethylglutarate was described by Sekura et al. (1976) as an inhibitor of glutathione synthetase. SAPH-3 disulfide was shown to be

30  
35



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a potent inhibitor of glutathione synthetase (Schor et al., 1991). Suitable doses of 7,8-dihydrofolate, trimethoprim,  $\alpha$ -aminomethylglutarate and SAPH-3, for use in combination with antibiotics, will generally be  
5 between about 0.1 and about 200 mg/kg body weight/day, and preferably, between about 1.0 and about 75 mg/kg body weight/day.

## EXAMPLE VII

Inhibitors of Methionine Synthetase  
and Homocysteine Transmethylase

Nitrous Oxide ( $N_2O$ )

15 Yagiela (1991) reported that nitrous oxide reacts with the reduced form of vitamin  $B_{12}$ , consequently inhibiting methionine synthetase, an enzyme that directly supports methylation reaction and nucleic acid synthesis.  
20  $N_2O$  is commercially available from Aldrich Chemical Company, amongst other sources.

50%  $N_2O$  is usually used clinically. However, evidence exists that prolonged exposure to clinical  
25 concentrations of  $N_2O$  inhibits cellular proliferation and can lead to megaloblastic anemia, leukopenia, and thrombocytopenia (Yagiela, 1991). Continuous administration beyond 24 hours, or repeated  
administration more frequently than once every 3 to 4  
30 days, should therefore be avoided to prevent leukopenia and megaloblastic changes. Biochemical recovery from exposure begins once inhalation is stopped, and can be improved by folinic acid and analogues, methionine and  
vitamin  $B_{12}$ . For example, Ostreicher (1994) recently  
35 reported that vitamin  $B_{12}$  supplements reverse some of the effects of chronic  $N_2O$  exposure.

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It has been shown the certain bacteria, e.g., *Salmonella typhimurium* (Baden & Monk, 1981), *Streptococcus faecalis*, *E. coli*, and *S. aureus* are inhibited by N<sub>2</sub>O at elevated pressures. It is therefore  
5 expected that N<sub>2</sub>O, at currently used clinical levels and durations (as above), in combination with, e.g., an MLS antibiotic, will show increase antimicrobial killing. Alternatively N<sub>2</sub>O may be used at elevated pressure in combination with antibiotics.

10

## EXAMPLE VIII

Inhibitors of Adenosine Deaminase (ADA)

## Coformycin and Isomers

15

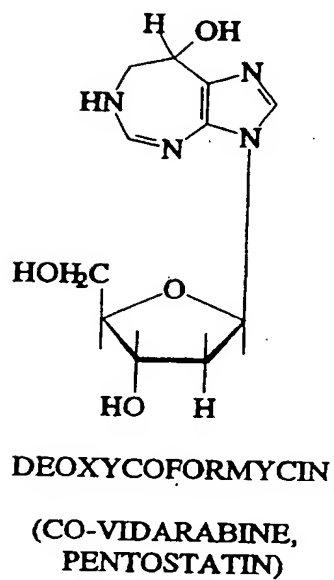
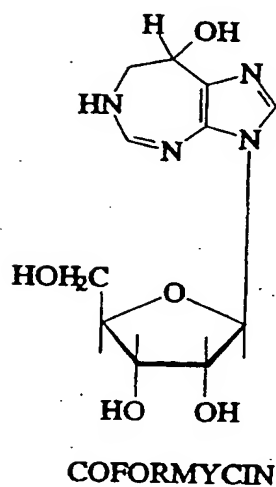
Adenosine deaminase inhibitors are also known to inhibit methylation indirectly by increasing the concentration of SAH. Coformycin and its 2'-deoxy isomer are naturally occurring potent inhibitors of ADA. Two  
20 further, chemically synthesized inhibitors of ADA are 1, 6-dihydro-6-hydroxy-methylpurine nucleoside and erythro-9-(2-hydroxy-3-nonyl)adenine (Suhadolnik, 1979). These four ADA inhibitors are shown below:

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5

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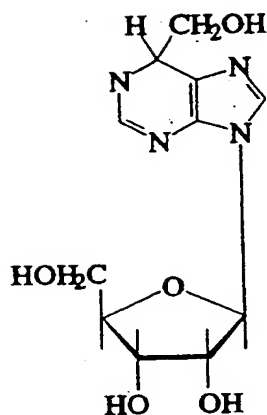
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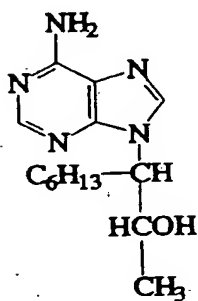


1, 6-DIHYDRO-6-HYDROXY-  
METHYLPURINE  
NUCLEOSIDE

15

20

25



ERYTHRO-9-(2-  
HYDROXY-3-NONYL)-  
ADENINE

30

ADA inhibitors show anti-tumor and immunosuppression activities (Suhadolnik, 1979). Lindley & Pisoni (1993) showed that conformycin (2.5 mM), deoxyconformycin (0.02 mM), 2'-deoxyadenosine (2.5 mM), 6-methylaminopurine riboside (2.5 mM),

35

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2'-3'-isopropylidene-adenosine (2.5 mM) and erythro-9-(2-hydroxy-3-nonyl)adenine (0.2 mM) inhibited lysosomal ADA by > 97%. O'Dweyer et al. (1988) showed that 2'-deoxyconformycin (pentostatin), has striking  
5 antitumor activity. Pharmacological studies by these investigators led to the definition of a safe and effective low weekly dose, at which protracted ADA inhibition occurs in neoplastic cells. This dose is 0.13 mg/kg. They also reported that patients tolerated 1  
10 mg/kg/day for 5 days with minimal distress. Brogden et al. (1993) determined the intravenous therapeutic dosage of pentostatin for lymphoblastic leukemia to be between 0.1 to 1 mg/kg/day.

15 Pentostatin has received FDA labeling approval for the treatment (3-6 month) of hairy cell leukemia in combination with alpha interferon (Kane et al., 1992). Based on these results, it is expected that dosages similar to the approved clinical dosages of pentostatin,  
20 namely 0.1 to 1 mg/kg/day, in combination with the antibiotics, will effectively kill bacteria.

#### EXAMPLE IX

##### Inhibitors of Dihydrofolate Reductase (DHFR)

25

##### **Methotrexate (MTX)**

Nesher et al. (1991) showed that, *in vitro*, low concentrations of methotrexate (MTX) interfere with  
30 specific methylation reactions in peripheral blood monocytes.

Svardal et al. (1988) suggested that MTX affects SAH metabolism in both cultured cells and patients and this  
35 may be explained by a lack of the 5-

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methylnetetrahydrofolate required for salvage of SAH to methionine.

MTX is used clinically at dosages of either 2.5 to 5.0 mg/day or 25 to 50 mg/single dose/week (Berkow et al., 1992) and it is polyglutamated in vivo. It is proposed that MTX at such clinically relevant dosages in combination with antibiotics will kill bacteria. It was shown by the inventors that when MTX (0.018 mM) was evaluated with the first procedure it was shown to enhance the relative activity of erythromycin against *S. aureus* 27660 by 233% (Table 8).

It is also contemplated that aminopterin could be used similarly to MTX.

#### Trimethoprim

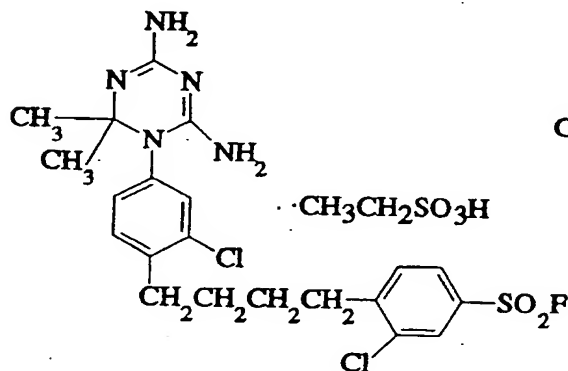
Roth (1986) indicated that trimethoprim, which retains only the 2,4-diaminopyrimidine moiety of MTX, retains high inhibitory activity only for bacterial DHFR. This inhibition therefore prevents tetrahydrofolate-dependent transmethylation reactions in bacteria (Schmidt et al., 1977), which is an important finding. From results such as these the inventors propose that trimethoprim and analogues could be used in combination with, e.g., MLS antibiotics, to kill bacteria. The typical clinical trimethoprim dosage is about 100 mg/12 hr (Berkow et al., 1992), and similar doses are proposed for use with the present invention. The inventors have shown in FIG. 2 that trimethoprim (0.26 mM) pre-mixed with agar inhibits *S. aureus* 27660 (resistant strain) 275% relative to control.

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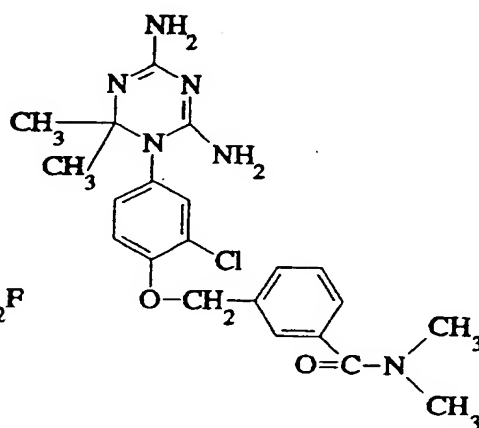
**4,6-Diamino-2,2-dimethyl-s-triazines**

Numerous 4,6-Diamino-2,2-dimethyl-s-triazines analogues have been synthesized especially in the laboratory of Baker (Baker & Vermeulen, 1969, 1970a, 1970b) and shown to be excellent inhibitors of DHFR. One such analogue was evaluated by Corbett et al. (1982) as being highly active against four transplantable colon adenocarcinomas in mice.

Based on these results it is expected that 3-chloro-4-{4-(2-chloro-4-[4,6-diamino-2,2-dimethyl-s-triazin-1(2H)-yl)]butyl}benzenesulfonyl chloride (NSC 127755, shown below) and 3-chloro-4-{4-(2-chloro-4-[4,6-diamino-2,2-dimethyl-s-triazin-1(2H)-yl]phenoxy)methyl}-N,N-dimethylbenzamide (NSC 139105, shown below) in combination with antimicrobial agents such as MLS antibiotics will kill microorganisms. Since the s.c. toxicity in mice was determined as  $\geq 156$  mg/kg ( $LD_{100}$ ), it is expected that NSC 127755 and analogues will be used at concentrations of about 1 to 100 mg/kg body weight and preferably at about 1 to 50 mg/kg.



NSC 127755  
TRIAZINE ANTIFOLATE

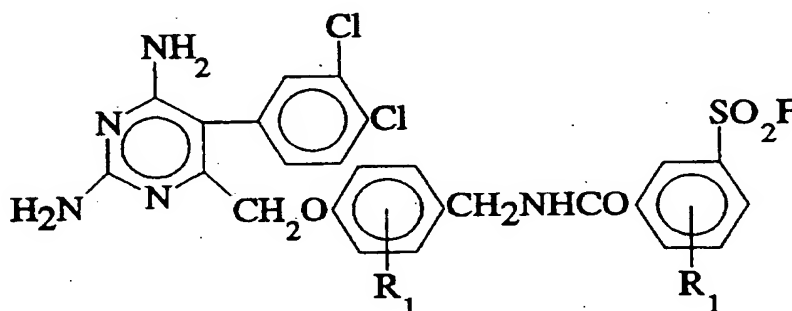


NSC 139105  
BAKER'S ANTIFOL

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**2,4-Diamino-5-(3,4-dichlorophenyl)pyrimidines**

Baker & Vermeulen (1969; 1970b) also described inhibitors of this group. It is expected that inhibitors of this group in combination with MLS antibiotics will kill microorganisms at dosages similar to that of the 4,6-diamino-2,2-dimethyl-s-triazines described above.


$$R_1 = R_2 = H$$
$$R_1 = H; R_2 \neq H$$
$$R_1 \neq H; R_2 = H$$
**Further Inhibitors**

Numerous inhibitors of DHFR are known in the literature (Roth, 1986; Bowden et al., 1993; Fleming & Schilsky, 1992). Blaney et al. (1984) reviewed over 1700 inhibitors of DHFR known at that time. To compare the inhibitors synthesized in numerous laboratories, these authors have reported the results in terms of either log 1/C or log K<sub>iapp</sub>. The potency of inhibitors were calculated from reported I<sub>50</sub> or K<sub>i</sub> values and expressed as either log 1/C or log K<sub>iapp</sub>. No distinction was made between these two parameters in this publication. The semi-quantitative nature of these parameters are suitable to indicate the approximate potency of the reported



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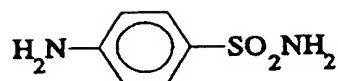
preferably >5, will work in combination with antibiotics to kill bacteria and microorganisms.

5 Such inhibitors are contemplated for use in combination with antibiotics at doses of between about 0.1 and about 200 mg/kg body weight/day, and preferably, between about 1 and about 100 mg/kg body weight/day.

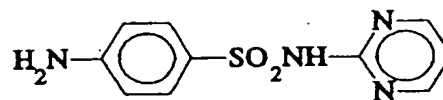
10 **Inhibitors of Dihydropteroate Synthetase: Sulfonamides**

Bacteria cannot utilize preformed folic acid. Sulfonamides function as competitive inhibitors of p-aminobenzoic acid (PABA) incorporation into dihydropteroic acid, the immediate precursor of folic acid. Sensitive organisms are those that must synthesize their own folic acid. Mammalian cells are not effected since they required preformed folic acid. In addition, sulfonamides combine with the pteridine moiety to deplete the system of pteridine if PABA is not present (Madell & Sande, 1990). The structures of sulfanilamide, sulfadiazine, sulfamethoxazole, sulfisoxazole and sulfacetamide are shown below:

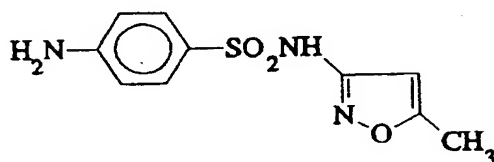
- 132 -



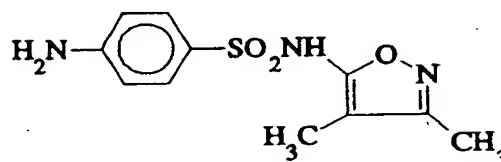
Sulfanilamide



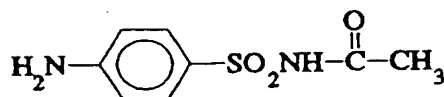
Sulfadiazine



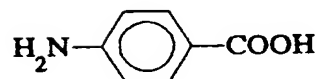
Sulfamethoxazole



Sulfisoxazole



Sulfacetamide



Para-aminobenzoic Acid

Clinical doses typically range from 2 to 8 g/day for sulfisoxazole as a typical example. Trimethoprim, a DHFR inhibitor is generally used in a combination with sulfamethoxazole, a dihydropteroate synthetase inhibitor. This combination is important clinically for a number of microorganisms and is used at dosages of about 800/160 mg/day (SMX/TMP; Berkow et al., 1992).

Sulfadiazine (0.056 M) was evaluated by the inventors using the first described disc susceptibility procedure and was shown to enhance the relative activity of erythromycin against *S. aureus* 27660 by 183% (Table 8).

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## EXAMPLE X

Inhibitors of Polyamine Synthesis

Igarashi et. al. (1979) showed a decrease in polypeptide synthesis when an *E. coli* polyamine-requiring mutant was grown in the absence of polyamines. This suggested to the inventors that when inhibitors of polyamine synthesis are combined with antimicrobials, increased antimicrobial activity should be observed, due to the reduction in methylase synthesis. A number of agents capable of inhibiting polyamine synthesis are described below.

 $\alpha$ -methylornithine

Mach et al. (1982) reported that polyamines are essential in prokaryotes and eukaryotes for optimal growth and development. Polyamines had previously been reported to bind in a specific way to both tRNAs and ribosomes. Mach et al. (1982) presented data to show that SAM-dependent transmethylation reactions can be regulated by alterations of polyamine concentrations in vivo. Methyltransferases from *Dictyostelium discoideum* were almost inactive without the addition of added polyamines. Additionally, for example, it was found that the growth of *D. discoideum* was arrested in four hours with 5 mM  $\alpha$ -methylornithine.

$\alpha$ -methylornithine is available from Sigma Chemical Company, and is proposed for use in combination with antibiotics at doses of between about 1 and about 1000 mg/kg body weight/day, and preferably, between about 10 and about 200 mg/kg body weight/day.

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**1,3-diaminopropan-2-ol**

In the studies by Mach et al. (1982), the growth of *D. discoideum* was also arrested in four hours using 5 mM 1,3-diaminopropan-2-ol. This compound is commercially available from Fluka (Buchs, Switzerland), and is also proposed for use at doses of between about 1 and about 1000 mg/kg body weight/day, and preferably, between about 10 and about 200 mg/kg body weight/day.

**Difluoromethylornithine and Difluoromethylarginine**

Tyms et al. (1988) reviewed the use of polyamine inhibitors in antimicrobial chemotherapy. Concerning difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), they indicated that some bacteria may not show growth inhibition since an alternative biosynthetic pathway for putrescine exists in these organisms.

To block putrescine biosynthesis in some procaryotic cells using DMFO, inhibition of an additional pathway is necessary. This can be achieved by the prevention of arginine formation through inhibition of arginine decarboxylase activity, e.g., using difluoromethylarginine (DFMA), another catalytically activated, irreversible inhibitor.

It has been shown in clinical trials that DFMO is effective against Trypanosomiasis at different dosage levels and modes of treatment by different groups. As an example, DFMO was administered in one study at 400 mg/kg qid, for 4 to 6 weeks. In another study, DFMO was administered 20g i.v. for 11 days, 30 g daily for 2 days and 15g orally every day for 7 weeks. Tyms (1988) concluded that DFMO is safe and well tolerated and the

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side effects are reversible. Additionally, DL- $\alpha$ -monofluoromethyldehydroornithine methylester, a DFMO analogue, is taken up more readily than DFMO ( $K_i=39 \mu\text{M}$ ) and readily cleaved to the free amino acid within the cell and has a  $K_i$  of  $3 \mu\text{M}$ .

The present inventors therefore propose that DFMO, DFMA or a combination of DFMO and DFMA, will be effective as inhibitory agents, or as an inhibitory package, for use with an antimicrobial agent, such as an MLS antibiotic. It is proposed that DFMO or analogues alone or in combination with DFMA could be used successfully with antimicrobials to kill microorganisms at doses of about 1 to 10,000 mg/kg per day, and preferably 50 to 1000 mg/kg/day. DFMO (0.046 M) was evaluated by the inventors using the first disc susceptibility procedure and shown to enhance the relative activity of erythromycin against *S. aureus* by 250% (Table 8).

#### **Inhibitors of S-Adenosylmethionine Decarboxylase (SAM-DC)**

The potential importance of inhibitors of the first enzyme in the synthesis of polyamines have been demonstrated by the success of DFMO, an inhibitor of ornithine decarboxylase, in the treatment of *Trypanosomiasis*. In order to convert putrescine into polyamines spermine and spermidine, the aminopropyl group is supplied by the catalytic activity of SAM-DC. Therefore, the action of SAM decarboxylase is essential for the synthesis of polyamines. It is therefore expected that inhibitors of SAM decarboxylase will operate in a similar fashion to ornithine decarboxylase inhibitors.

Pegg & McCann (1992) suggested that SAM-DC inhibitors may be of therapeutic value, either alone or

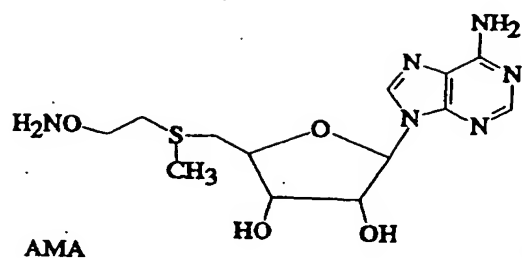
- 136 -

in combination with ornithine decarboxylation inhibitors. These authors described powerful irreversible inhibitors of SAM-DC that are available, including 5'-([ (Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine, an enzyme activated inhibitor, and 5'-deoxy-5'-[(3-hydrazinopropyl)methylamino]adenosine, which binds to the active site and forms a covalent bond with the pyruvate prosthetic group. The teachings of Pegg & McCann (1992) regarding SAM-DC inhibitors in the treatment of tumors and, particularly, in the treatment of protozoan parasites, is relevant to the choice of SAM-DC inhibitors and appropriate doses for use in the combined antimicrobial therapy of the present invention.

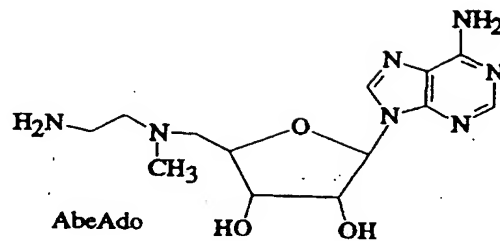
A number of useful inhibitors of SAM-DC have been developed based on substrate or product analogues or compounds that form a covalent bond with the pyruvate co-factor. Some of the important inhibitors are AMA (S-(5'-deoxy-5'-adenosyl)methylthioethylhydroxylamine), MHZPA (5'-deoxy-5'-[(3-hydrazinopropyl)methylamino]adenosine), MAOEA (5'-deoxy-5'-[(2-aminooxyethyl)-methylamino]adenosine, AbeAdo (5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine [MDL 73811]), AdoMac (S-(5'-deoxy-5'-adenosyl)-1-amino-4-methylthio-2-cyclopentene, MGBG (methylglyoxal bis(guanyl)hydrazone), CGP-39'937 and CGP-33'829.

Pegg & McCann (1992), reported that AbeAdo is at least 100-fold more potent than DFMO, which is now an approved drug for West African sleeping sickness. It is proposed that AbeAdo and analogues alone, or in combination with DFMA and or DFMO, could be used successfully with antibiotics to kill bacteria. The proposed dosages of AbeAdo and analogues is from about 1 to about 1,000 mg/kg per day, and preferably, from about 1 to about 200 mg/kg/day.

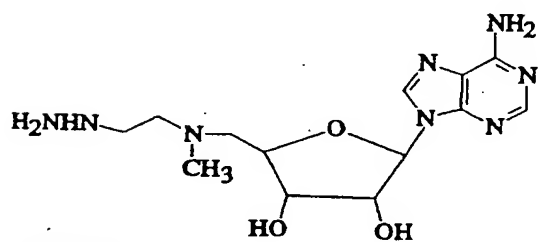
- 137 -



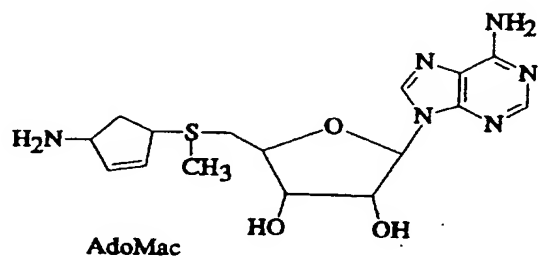
AMA



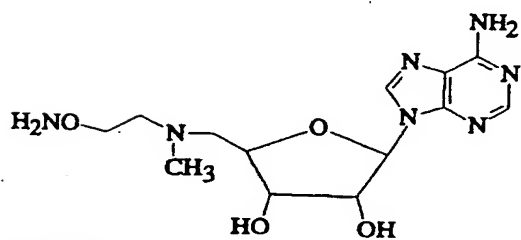
AbeAdo



MHZPA

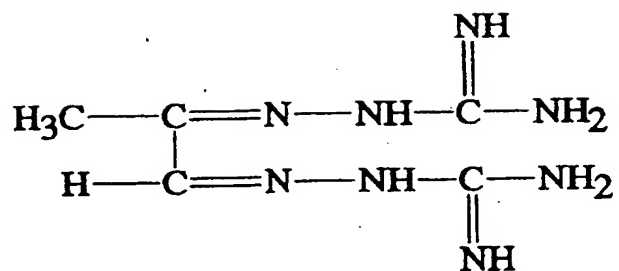


AdoMac



MAOEA

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### Inhibitors of Ornithine Decarboxylase (ODC)

McCann & Pegg (1992) reported that ornithine  
5 decarboxylase (ODC) is a suitable enzyme target for use  
in certain therapeutic regimens. These authors describe  
the therapeutic effects resulting from ODC inhibition and  
particularly describe the inhibitor, eflornithine.  
Recent *in vivo* therapeutic approaches to ODC inhibition  
10 are also described, as may be applied cancer treatment,  
cancer chemoprevention, autoimmune diseases, ischemia,  
hyperplasia, hearing loss, African trypanosomiasis, AIDS,  
and other infectious diseases/organisms. In light of the  
teachings of McCann & Pegg (1992), and the present  
15 disclosure, the use of ODC inhibitors can be readily  
adapted for combination therapy with antimicrobials in  
the treatment of microbial infections and diseases.

### Inhibitors of Arginine Decarboxylase (ADC)

20 As stated above, Tyms et al. (1988) reported on the  
inhibition of polyamine synthesis by preventing arginine  
formation. This is achieved through the inhibition of  
arginine decarboxylase (ADC). DFMA is a irreversible  
25 inhibitor of ADC, which is contemplated for use in the  
present invention at similar doses to those quoted above  
for DFMO. Kallio et. al. (1981) described  
DL- $\alpha$ -(difluoromethyl)arginine as a potent and  
irreversible inhibitor of bacterial ADC.  
30 DL- $\alpha$ -(difluoromethyl)arginine (RMI 71669) will thus be  
effective alone and also in combination with ODC  
inhibitors. In the latter case, all putrescine  
biosynthesis may be inhibited in prokaryotic cells.

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## EXAMPLE XI

Inhibitors of RNA Maturation

## 5-Azacitidine

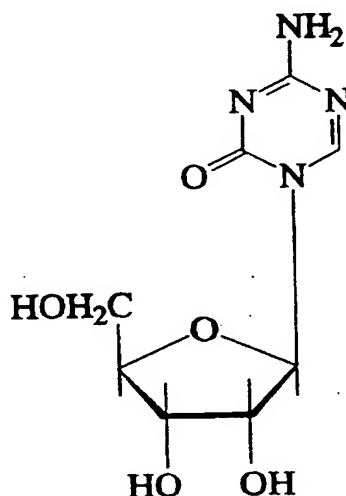
5

5-Azacitidine inhibits the maturation of rRNA (Suhadolnik, 1979). The formation of 28S and 18S, but not of 38S RNA, is severely inhibited. 5-Azacitidine is commercially available from Sigma Chemical Company. The structure of 5-Azacitidine is shown below. Budavari et al. (1989) reported the oral toxicity of 5-azacitidine as 572.3 mg/kg in mice. This compound is therefore proposed for use with antibiotics at doses of between about 1 and about 300 mg/kg body weight/day, and preferably, between about 10 and about 75 mg/kg body weight/day.

20

25

30



## Cordycepin (3'-deoxyadenosine)

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Cordycepin (Suhadolnik, 1979) inhibits the synthesis of mature rRNA, ribosomal precursor 45S RNA and tRNA in

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Hela cells; DNA and protein synthesis are not affected. Cordycepin at 200  $\mu\text{g/ml}$  totally inhibits RNA synthesis and at lower concentrations, rRNA and tRNA syntheses are selectively inhibited, but protein synthesis is not effected. Cordycepin is commercially available from Sigma Chemical Company and it's structure is shown below. It is proposed for use with antibiotics at doses of between about 1 and about 250 mg/kg body weight/day, and preferably, between about 5 and about 75 mg/kg body weight/day.

#### Toyocamycin and Analogues

Suhadolnik (1979) indicated that tubercidin, toyocamycin and 6-thioguanosine inhibit the formation of mature 28S and 18S rRNA in Novikoff hepatoma cells. Similarly, toyocamycin was reported to inhibit the maturation of rRNA in *S. cerevisiae*, and is proposed for use with, e.g., MLS antibiotics, at doses of between about 0.1 and about 100 mg/kg body weight/day, and preferably, between about 0.1 and about 50 mg/kg body weight/day.

#### EXAMPLE XII

##### ANTISENSE OLIGONUCLEOTIDES

Cohen & Hogan (1994; incorporated herein by reference) describe the general approach used to develop synthetic oligonucleotides as drugs.

#### Translation Inhibition

DNA or RNA antisense oligonucleotides are also contemplated as another means of inhibiting bacterial methylation. In such methods, oligonucleotides capable of hybridizing with those portions of the mRNA sequences

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that are involved in binding to the ribosome or to the methylation site of the rRNA may be employed. This will block the induction of the bacterial methylase synthetase and thus reduce antibiotic resistance when used in combination with an antibiotic. Exemplary sequences are set forth below, modifications of which may be readily designed using the teaching herein and the known sequences and structural information, such as that described by Dubnau (1984; 1985) and Denoya et al. (1986).

*ermC* is known to specify an rRNA methyltransferase that confers resistance to erythromycin. The transferase N<sup>6</sup>,N<sup>6</sup>-dimethylates an adenine residue at position 2058 on the 23S rRNA, thereby decreasing ribosomal affinity for erythromycin. Basically the *ermC* mRNA exists in an inactive conformation in which the ribosome binding site for methylase synthesis, the Shine Delgarno sequence 2 (SD2), is sequestered by base pairing. Stalling of a ribosome under the influence of erythromycin binding during translation of a 19-amino acid leader peptide causes the inactive mRNA structure to open, and free the sequestered SD2 permitting initiation of methylase synthesis.

The structure of the *ermC* mRNA regulatory region is shown in FIG. 1A and FIG. 1B of Dubnau (1985) and FIG. 3 of Dubnau (1984). In this region, the ribosomal binding site for the putative leader peptide is defined as SD1. SD1 has the sequence AGGAGGA, which is followed six residues down by the initiation codon ATG (also referred to as AUG). The complete SD1 site, AGGAGGAAAAATATG (SEQ ID NO:1), is the primary target proposed for antisense inhibition.

35

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The inventors have designed complementary oligonucleotide sequences against this region that will hybridize to the SD1 + initiation codon region, thus preventing binding of this mRNA to the ribosome. A candidate complementary, or antisense, oligonucleotide is: TCCTCCTTTTTTATAC (SEQ ID NO:2), or UCCUCCUUUUUUUAUAC (the ribooligonucleotide, SEQ ID NO:3).

In the *ermC* mRNA region, there is also an additional ribosomal binding site, SD2. This has a regulatory role and is usually translationally inactive due to base pairing. SD2 and the initiation codon has the sequence AGAGGGTTATAATG (SEQ ID NO:4). Since the *ermC* mRNA structure in the regulatory region can re-arrange into different conformations, and thus open the SD2 sequence, an oligonucleotide complementary to the SD2 + initiation codon region is also proposed for use in arresting translation of the methylase enzyme. An appropriate candidate antisense deoxy oligonucleotide is: TCTCCCAACACTAC (SEQ ID NO:5).

Oligonucleotides will be designed and synthesized (Gait, 1984; Zon & Geiser, 1991). The appropriate length of the oligonucleotide to allow efficient hybridization at physiological salt concentration and temperature will be determined by computer analysis (Rychlik & Rhoads, 1989; Van Ness & Chen, 1991). These oligonucleotide will contain appropriate modifications that will allow sufficient stability *in vivo* and allow adequate transport into the cell. These oligonucleotide will be evaluated *in vitro* by their ability to prevent synthesis of the rRNA methylase. Active compound identified *in vitro* *in vivo* will then be evaluated *in vivo* as a "new antibiotic", as described by Lorian (1991).

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**Decoy Substrates**

Oligoribonucleotides with sufficient stability are also contemplated for use as decoys to serve as  
 5 substrates for the methylase enzyme. The "decoy oligos" would have tight binding properties and function to bind with, and saturate, the active-site of the methylase enzyme. Appropriate decoy sequences are those surrounding the target site of dimethylation on the 23S  
 10 rRNA and the autoregulatory site on the *ermC* mRNA. The oligos used may have the sequences as taught below or variations thereof designed using this disclosure and the sequences and structural information described in the literature, e.g., FIG. 6 of Denoya et al. (1986;  
 15 incorporated herein by reference).

Particular inhibitory structures proposed by the inventors are set forth below and are represented by SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8. Binding to the  
 20 double stranded sequence is preferred. The sequence will depend on the organisms targeted and may contain degenerated sequences. These structures can also contain any group or modification that the enzyme will tolerate.

25                   A    C  
                   GCGAC GGA GGAAAGACCCC  
                   CGCUG CCU CC       GGGG  
                           AU

30    and

                  A    C  
                   GCGAC GGA GGAAAGACCCC

35       Additionally, the potency of the decoy oligos can be improved by designing a group into the molecule that has the ability to covalently attach to the enzyme and permanently inactivate it. Many different groups that function in this capacity are known in the art. For

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example, the reactive oligonucleotide derivatives used as gene-targeted biological active compounds, that are currently in use in this field, have recently been reviewed Knorre & Vlassov (1991). These authors  
5 indicated that since it appears that binding of deoxyribonucleotides to the coding region of mRNAs does not effect translation, because the translating ribosome displaces bound oligonucleotides, coupling of reactive groups that crosslink to form covalent bonds with the  
10 target will create an insurmountable block for the ribosome. Examples of modifications coupled to oligonucleotides that crosslink to target are 2-chlorethylamino-, EDTA Fe- and o-phenanthroline Cu-groups. Additionally groups that increase binding to  
15 nucleic acid targets could also be used, such as phenazinium-conjugated oligonucleotides.

#### Specific Bacterial Targets

20 It will be understood that, generally, the sequence of methylase mRNAs and rRNAs from different bacteria will not be identical. The sequence variations will need to be considered when designing oligonucleotides for use in connection with specific bacteria. Sequences are readily  
25 available from data bases, such as GenBank.

#### Modifications

30 Depending on the  $T_m$  of the oligonucleotide:mRNA hybrid, the length of the oligonucleotide will be optimized for maximum activity.

In addition to any of the common bases, the oligonucleotides may also contain other naturally  
35 occurring or modified bases. For example, both deoxyribonucleotides and ribonucleotides may contain

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backbone modifications, including methylphosphonates, phosphorothioates, phosphorodithioates and PNAs (protein nucleic acids; sugar modifications, including 2'-alkyl groups; base modifications, including inosine, 5 alphanucleoside, and 3' and 5' blocking groups, including cholesterol, 3' and 5' alkylhydroxyl groups, and interchelators.

Such modifications, individually or in combination, 10 are proposed for use in designing an oligonucleotide that hybridizes specifically to the initiation target and additionally contains sufficient stability against nuclease degradation. Certain preferred oligonucleotides include 3'- and/or 5'-blocked oligodeoxynucleic acids. 15 The advantage of such oligodeoxynucleotides is that the endogenous RNase H, would degrade the complementary mRNA when the oligodeoxynucleotide/mRNA hybrid. This is contemplated to allow permanent inhibition of the translation of this particular methylase mRNA.

20

#### Oligonucleotide Delivery

A technology that allows the transport of oligonucleotides into bacteria has recently been 25 developed (Couvreur et al., 1991; incorporated herein by reference) and is commercially available from Genta Corporation, San Diego, CA.

#### EXAMPLE XIII

30

#### PHARMACEUTICAL COMPOSITIONS

Compositions of the present invention may comprise a combined effective amount of an antimicrobial agent or antibiotic and an agent that inhibits rRNA methylation or 35 maturation, or such agents may be provided as distinct compositions, intended for use in combination. The



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antibiotics, second agent inhibitors or combinations thereof, may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium, or may be provided in solid form.

5

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic or other untoward reactions when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, additional antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like (USP XXII, The United States Pharmacopeia, 1990; incorporated herein by reference).

15

The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients, particularly other non-MLS antibiotics, can also be incorporated into the compositions.

25

In addition to the detailed information provided herein, the teachings of Eliopoulos & Moellering (1991) and Shungu (1991) are incorporated by reference for the purposes of further describing appropriate antimicrobial combinations and the use of antibiotics in medicine.

30

Kits for use in the present invention are contemplated to include various combinations of solutions, tablets, ointments and other formulations, and also to encompass two component systems. One component will be at least one inhibitor that will be administered,

35

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generally at a predetermined time before the MLS antibiotic (the second component). Additional components may be used in the context of the therapeutic methods provided by this invention, although they may not be included in a kit *per se*. For example, N<sub>2</sub>O, used extensively in dentistry, may be employed in essentially the same way, and given simultaneously and/or as a pre-treatment to antibiotic therapy.

#### 10 Parenteral Formulations

The active compounds, whether antibiotics, second agent inhibitors, or combinations thereof, may be formulated for parenteral administration, e.g., formulated for injection via the intravenous, intramuscular, sub-cutaneous, or even intraperitoneal routes (USP XXII, The United States Pharmacopeia, 1990). The preparation of an aqueous composition that contains such active ingredients, or combinations thereof, will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared, and the preparations can also be emulsified.

Solutions of each or both of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant (USP XXII, The United States Pharmacopeia, 1990) such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, in mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations will contain a preservative to prevent the growth of microorganisms.

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The pharmaceutical forms suitable for injectable use include: aqueous solutions or dispersions, formulations including sesame oil, peanut oil or aqueous propylene glycol and powders for the extemporaneous preparation of injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The antibiotics and second inhibitory agents can be formulated into one or more compositions in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, lactic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example,

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parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more, or highly, concentrated solutions for intramuscular injection is also contemplated. This is envisioned to have particular utility in cases where either the antibiotic or the inhibitor is sensitive to digestive conditions. In this regard, the use of DMSO as solvent is preferred as this will result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as are therapeutically effective, as outlined

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in the foregoing examples. The formulations are easily administered in a variety of dosage forms. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if  
5 necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection,  
10 sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the  
15 proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for  
20 administration will, in any event, determine the appropriate dose for the individual subject.

In addition to the compounds formulated for parenteral administration, other pharmaceutically  
25 acceptable forms are also contemplated for use, including, e.g., tablets or other solids for oral administration; time release capsules; forms for topical administration, including creams and lotions; mouthwashes; aerosols, inhalents and the like.

30 The components for use with the invention may be prepared in the form a treatment kit wherein the antibiotic and inhibitory agent are located within a suitable container, such as at least one vial, test tube,  
35 flask, bottle, syringe or other container, into which the active components are placed, and preferably, suitably

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allocated. Where desired, the antibiotic and inhibitor may be provided in distinct containers, such that the kit will contain two vials, or other containers, each of which contains only one of the active agents. These  
5 "therapeutic kits" will also typically include a means for containing the vials or the containers in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials are retained.

10

#### Oral Formulations

In certain embodiments, active compounds may be administered orally. This is contemplated for agents  
15 that are generally resistant, or have been rendered generally resistant, to proteolysis by digestive enzymes. Such compounds are contemplated to include the antibiotics and most of the inhibitory second agents disclosed herein. Naturally, the preferred inhibitors  
20 will be the more active compounds and those already cleared by the FDA for other uses, as will be known to those of skill in the art in light of the present disclosure.

25 For oral administration, the active compounds and/or combinations thereof may be administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or compressed into tablets, or  
30 incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such  
35 compositions and preparations should contain at least 0.1% of active compounds or combinations thereof. The

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percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings to modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

### Topical Formulations

The formulation of antimicrobial agents, such as antibiotics, and second, inhibitory agents for topical use, such as in creams, ointments and gels is also contemplated. The preparation of oleaginous or water-

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soluble ointment bases is also well known to those in the art. For example, these compositions may include vegetable oils, animal fats, and more preferably, semisolid hydrocarbons obtained from petroleum.

5 Particular components used may include white ointment, yellow ointment, cetyl esters wax, oleic acid, olive oil, paraffin, petrolatum, white petrolatum, spermaceti, starch glycerite, white wax, yellow wax, lanolin, anhydrous lanolin and glyceryl monostearate. Various  
10 water-soluble ointment bases may also be used, including glycol ethers and derivatives, polyethylene glycols, polyoxyl 40 stearate and polysorbates. Even delivery through the skin may be employed if desired, e.g., by using transdermal patches, ionophoresis or  
15 electrotransport. Examples of hydrophilic and hydrophobic ointments are known in the art, (e.g., USP XXII, The United States Pharmacopeia, 1990).

#### Nasal Solutions and Inhalants

20

One may also use nasal solutions or sprays, aerosols or inhalants with the antimicrobials and second agents described herein. Nasal solutions are usually aqueous solutions designed to be administered to the nasal  
25 passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and slightly buffered to maintain a pH of  
30 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, and appropriate drug stabilizers, if required, may be included in the formulation. Various commercial nasal preparations are known and include, for example, those  
35 for use with various antibiotics and antihistamines.



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Inhalations and inhalants are pharmaceutical preparations designed for delivering a drug or compound into the respiratory tree of a patient. A vapor or mist is administered and reaches the affected area to give relief from symptoms of bronchial and nasal congestion. Inhalations may be administered by the nasal or oral respiratory routes. The administration of inhalation solutions is only effective if the droplets are sufficiently fine and uniform in size so that the mist reaches the bronchioles.

Another group of products, also known as inhalations, and sometimes called insufflations, consists of finely powdered or liquid drugs that are carried into the respiratory passages by the use of special delivery systems, such as pharmaceutical aerosols, that hold a solution or suspension of the drug in a liquefied gas propellant. When released through a suitable valve and oral adapter, a metered dose of the inhalation is propelled into the respiratory tract of the patient.

Particle size is of major importance in the administration of this type of preparation. It has been reported that the optimum particle size for penetration into the pulmonary cavity is of the order of 0.5 to 7  $\mu\text{m}$ . Fine mists are produced by pressurized aerosols and hence their use is considered advantageous. The active compounds described above, whether antibiotics, second agent inhibitors, or combinations thereof, may be formulated for nasal administration in any of these methods.

#### Ophthalmic Solutions

The antimicrobials and agents that inhibit rRNA methylation or maturation may also be used in

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preparations of pharmaceutical compositions suitable for use as ophthalmic solutions. The preparation of ophthalmic formulations in accordance with conventional pharmaceutical practice is well known, see for example  
5 "Remington's Pharmaceutical Sciences" 15th Edition, pages 1488 to 1501 (Mack Publishing Co., Easton, PA).

The ophthalmic preparations may contain antibiotics and/or second agents, or pharmaceutically acceptable  
10 salts thereof, in a concentration from about 0.01 to about 1% by weight, preferably from about 0.05 to about 0.5% in a pharmaceutically acceptable solution, suspension or ointment. Some variation in concentration will necessarily occur, depending on the particular  
15 compound employed, the condition of the subject to be treated and the like, and the person responsible for treatment will determine the most suitable concentration for the individual subject. The ophthalmic preparation will preferably be in the form of a sterile aqueous  
20 solution containing, if desired, additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents and the like.

25 Suitable preservatives for use in such a solution include benzalkonium chloride, benzethonium chloride, chlorobutanol, thimerosal and the like. Suitable buffers include boric acid, sodium and potassium bicarbonate,  
30 sodium and potassium borates, sodium and potassium carbonate, sodium acetate, sodium biphosphate and the like, in amounts sufficient to maintain the pH between about 6 and 8, and preferably, between about pH 7 and pH 7.5. Suitable tonicity agents are dextran 40, dextran  
35 70, dextrose, glycerin, potassium chloride, propylene glycol, sodium chloride, and the like, such that the

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sodium chloride equivalent of the ophthalmic solution is in the range 0.9 plus or minus 0.2%. Suitable antioxidants and stabilizers include sodium bisulfite, sodium metabisulfite, sodium thiosulfate, thiourea and the like. Suitable wetting and clarifying agents include polysorbate 80, polysorbate 20, poloxamer 282 and tyloxapol. Suitable viscosity-increasing agents include dextran 40, dextran 70, gelatin, glycerin, hydroxyethylcellulose, hydroxymethylpropylcellulose, lanolin, methylcellulose, petrolatum, polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone, carboxymethylcellulose and the like. The ophthalmic preparation will be administered topically to the eye of the subject in need of treatment by conventional methods, for example in the form of drops or by bathing the eye in the ophthalmic solution.

#### **Liposomes and Nanoparticles**

Couvreur et al. (1991) review the potential of liposomes and nanoparticles in the targeted antibiotic therapy of intracellular bacterial infections and diseases. Nanoparticles can generally entrap antibiotics in a stable and reproducible way (Henry-Michelland et al., 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1  $\mu\text{m}$ ) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are proposed for use in the present invention. They are easily made, as described by Couvreur et al. (1984; 1988). In serum, the liberation of ampicillin from nanoparticles was found to follow zero-order kinetics (Fattal et al., 1991a, 1991b).

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Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have  
5 diameters of from 25 nm to 4  $\mu$ m. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

10 Liposomes bear many resemblances to cellular membranes and are contemplated for use in connection with the present invention as carriers for the antibiotics and/or second agents. They are widely suitable as both water- and lipid-soluble substances can be entrapped,  
15 i.e., in the aqueous spaces and within the bilayer itself, respectively. It is possible that drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

20 The formation and use of liposomes is generally known to those of skill in the art. For example, Couvreur et al. (1991; incorporated herein by reference) describe the use of liposomes and nanoparticles in the  
25 targeted antibiotic therapy of intracellular bacterial infections and diseases. Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon & Papahadjopoulos, 1988; Allen & Choun, 1987).

30 In addition to the teachings of Couvreur et al. (1991), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when  
35 dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred

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structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures  
5 undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a  
10 characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble  
15 proteins such as cytochrome c bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations  
20 for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately  
25 efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are  
30 prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the  
35 physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar

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compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells via four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity and surface charge. They may persist in tissues for hours or days, depending on their composition, and half lives in the blood range from minutes to several hours. Larger liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue

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distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and tissues accessible to their large size. These  
5 include the blood, liver, spleen, bone marrow and lymphoid organs.

Targeting is generally not a limitation in terms of the present invention. However, should specific  
10 targeting be desired, methods are available for this to be accomplished. Antibodies may be used to bind to the liposome surface and to direct the antibody and its drug contents to specific antigenic receptors located on a particular cell-type surface. Carbohydrate determinants  
15 (glycoprotein or glycolipid cell-surface components that play a role in cell-cell recognition, interaction and adhesion) may also be used as recognition sites as they have potential in directing liposomes to particular cell types. Mostly, it is contemplated that intravenous  
20 injection of liposomal preparations would be used, but other routes of administration are also conceivable.

Using the combined antimicrobial formulations described herein in liposomal formulations will lead to  
25 further particular advantages that relate to targeting. The properties of liposomes and nanoparticles will likely improve therapy in a number of bacterial infections that are presently difficult to cure. As set forth by Couvreur *et al.* (1991), liposomes and nanoparticles  
30 themselves cannot escape from the circulation because of the endothelial barrier, with exception to tissues with discontinuous endothelia lining their capillaries (*i.e.*, liver, spleen, and bone marrow). This leads to the rapid clearance of these ultrafine particulate carriers from  
35 the blood and to their capture by the cells of the reticuloendothelial system (Poste, 1983; Grislain *et al.*,

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1983). This corresponds exactly to the same tissue distribution pattern as that of the majority of the bacteria responsible for intracellular infection. In addition, it has been shown that liposomes are taken up  
5 by circulating blood monocytes, which are known to infiltrate some infectious lesions (Tulkens, 1985). Also, as with bacteria, both liposomes and nanoparticles will likely penetrate cells by endocytosis, first forming phagosomes, which in turn fuse with lysosomes to form  
10 phagolysosomes or secondary lysosomes (Couvreur et al., 1977).

The advantages of liposomes and nanoparticles has been described for various cases. Liposome-associated  
15 cephalothin has been shown to be more effective than free drug in the treatment of experimental murine salmonellosis (Desiderio and Campbell, 1983). The therapeutic index of ampicillin, calculated on the basis of mouse mortality, has been reported to be increased by  
20 120-fold upon binding to nanoparticles (Fattal et al., 1989). Guinea pigs infected by *Brucella canis* and treated with liposome-entrapped streptomycin were found to be free of bacteria ( $2 \times 10$  mg/kg), whereas animals treated with the free drug, for the same schedule of  
25 administration, showed only a minor reduction in the number of surviving bacteria (Fountain et al., 1985). The delivery of antibacterial drugs to phagocytic cells has also been demonstrated to be feasible with amikacin (Düzgünes et al., 1988), kanamycin and tobramycin  
30 (Fountain et al., 1985).

#### Therapeutic Kits

Therapeutic kits comprising antimicrobial agents and  
35 methylation inhibitors are contemplated and will generally contain, in suitable container means, any of



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the previously described or other suitable pharmaceutically acceptable formulations of one or more antibiotics and inhibitors. The kit may have a single container means for both (or all) components or it may  
5 have more than one distinct container means.

When the components of the kit are provided in one or more liquid solutions, the liquid solution will generally be an aqueous solution, with a sterile aqueous  
10 solution being particularly preferred. However, the components may also be formulated into a syringable composition. In which case, the container means may itself be a syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied  
15 to an infected area of the body, injected into an animal, or even applied to and mixed with the other components of the kit.

The components of the kit may also be provided as  
20 dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means. The container means will generally  
25 include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antibiotics and methylation inhibitors may be placed, preferably, suitably allocated. The kits may also comprise the second/third container means mentioned above  
30 for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits of the present invention will also typically include a means for containing the vials in  
35 close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which

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the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention may also comprise, or be packaged with, an instrument for assisting with the administration, placement or injection of the components or combined composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle or device.

10

\* \* \*

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

30

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The following references, to the extent that they provide exemplary procedural or other details  
5 supplementary to those set forth herein, are specifically incorporated herein by reference.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

- (A) NAME: Oridigm Corporation
- (B) STREET: #6 Nickerson Street, Suite 201
- (C) CITY: Seattle
- 10 (D) STATE: Washington
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 98109

15

- (ii) TITLE OF INVENTION: Combinations and Methods for  
Reducing Antimicrobial  
Resistance

- (iii) NUMBER OF SEQUENCES: 8

20

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- 25 (D) SOFTWARE: PatentIn Release #1.0, Version  
#1.30 (EPO)

30

## (v) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: UNKNOWN
- (B) FILING DATE: CONCURRENTLY HEREWITH
- (C) CLASSIFICATION: UNKNOWN

## (vi) PRIOR APPLICATION DATA:

35

- (A) APPLICATION NUMBER: US 08/364,246
- (B) FILING DATE: 27-DEC-1994

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## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

10

AGGAGGAAAA ATATG

15

## (2) INFORMATION FOR SEQ ID NO: 2:

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## (i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 16 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

25

TCCTCCTTTT TTATAC

16

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 16 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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UCCUCCUUUU UUAUAC

16

## (2) INFORMATION FOR SEQ ID NO: 4:

5

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

10 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGAGGGTTAT AATG

15

14

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TCTCCCAACA CTAC

14

## 30 (2) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

35 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(6, 10)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "N = A, G, C, or T"

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCGACNGGAN GGAAAGACCC C

21

10

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

15

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(6, 10, 13-17)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "N = A, G, C, or T"

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGCUGNCCUN CCNNNNNGGG G

21

30

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

35

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

## (ix) FEATURE:

- 5 (A) NAME/KEY: modified\_base  
(B) LOCATION: one-of(6, 10)  
(D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "N = A, G, C, or T"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

10

GCGACNGGAN GGAAAGACCC C

21

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CLAIMS

1. A composition comprising an antimicrobial agent in  
5 combination with a second agent that inhibits methylation  
in a microorganism.

2. The composition of claim 1, wherein said  
10 antimicrobial agent is a macrolide lincosamide  
streptogramin B (MLS) antibiotic.

3. The composition of claim 2, wherein said MLS  
15 antibiotic is a macrolide antibiotic.

4. The composition of claim 3, wherein said MLS  
antibiotic is erythromycin, azithromycin, clarithromycin,  
20 roxithromycin, oleandomycin, spiramycin, josamycin,  
miocamycin, midecamycin, rosaramycin, troleandomycin,  
flurithromycin, rokitamycin or dirithromycin.

5. The composition of claim 2, wherein said MLS  
25 antibiotic is a lincosamide antibiotic.

6. The composition of claim 5, wherein said MLS  
30 antibiotic is lincomycin, clindamycin or celesticetin.

7. The composition of claim 2, wherein said MLS  
antibiotic is a streptogramin B antibiotic.

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8. The composition of claim 7, wherein said MLS antibiotic is pristinamycin or virginiamycin.

5 9. The composition of claim 1, wherein said second agent inhibits RNA methylation or maturation in a microorganism.

10 10. The composition of claim 9, wherein said second agent inhibits RNA methylation or maturation in a bacterium.

15 11. The composition of claim 10, wherein said second agent inhibits RNA methylation or maturation in an MLS-sensitive bacterium.

20 12. The composition of any one of claims 9-11, wherein said second agent inhibits RNA methylation.

25 13. The composition of claim 12, wherein said second agent inhibits an RNA methyltransferase.

30 14. The composition of claim 13, wherein said second agent is S-adenosylhomocysteine (SAH), an SAH analogue, homocysteine, an adenine derivative, a SAM analogue, a sulphonium SAM derivative, sinefungin, a sinefungin analogue, nicotinamide, a nicotinamide analogue or polyinosinate.

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15. The composition of claim 14, wherein said second agent is S-N<sup>6</sup>-methyladenosylhomocysteine, S-aristeromyciny-L-homocysteine, an S-aristeromyciny-L-homocysteine analogue, SIBA, S-Tubercidinylhomocysteine (STH), an STH analogue, A9145c, methylnicotinamide, MTA or xylosyladenine.

16. The composition of claim 12, wherein said second agent inhibits S-adenosylhomocysteine (SAH) hydrolase (SAHH).

17. The composition of claim 16, wherein said second agent is adenosine, an adenosine analogue, neplanocin A (NPA) or an NPA analogue.

18. The composition of claim 17, wherein said second agent is (S)-DPHA, (D)-eritadenine, (RS)-AHPA, adenosine dialdehyde, C<sup>3</sup>Ado, aristeromycin (Ari), an aristeromycin analogue, DHCeA, c<sup>3</sup>-DHCeA, DHCaA, c<sup>3</sup>-DHCaA, c<sup>3</sup>-NpcA, c<sup>3</sup>-Ari, 6'C-methylneplanocin A, 2'-deoxyadenosine, Ara-A or tubercidin.

25

19. The composition of claim 12, wherein said second agent inhibits S-adenosylmethionine (SAM) synthetase (SAMS).

30

20. The composition of claim 19, wherein said second agent is cycloleucine, a cycloleucine analogue, a methionine analogue or AMPNPP.

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21. The composition of claim 20, wherein said second agent is L-cis-AMB, L-cis-AMTB or 5' (R) - (C) - [(L-homocysteine-S-yl) methyl]adenosine 5' - ( $\beta, \gamma$ ) - imidotriphosphate.

5

22. The composition of claim 19, wherein said second agent inhibits glutathione synthetase.

10

23. The composition of claim 22, wherein said second agent is buthionine sulfoximine, 7,8-dihydrofolate,  $\alpha$ -aminomethylglutarate or SAPH-3.

15

24. The composition of claim 12, wherein said second agent inhibits methionine synthetase (MS) or homocysteine transmethylase.

20

25. The composition of claim 24, wherein said second agent is nitrous oxide ( $N_2O$ ).

25

26. The composition of claim 12, wherein said second agent inhibits adenosine deaminase (ADA).

30

27. The composition of claim 26, wherein said second agent is coformycin; a coformycin isomer, 1,6-dihydro-6-hydroxy-methylpurine nucleoside, erythro-9-(2-hydroxy-3-nonyl)adenine, 6-methylaminopurine riboside or 2'-3'-isopropylidene-adenosine.

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28. The composition of claim 12, wherein said second agent inhibits dihydrofolate reductase (DHFR).

5 29. The composition of claim 28, wherein said second agent is methotrexate (MTX), aminopterin, trimethoprim, a 4,6-diamino-2,2-dimethyl-s-triazine analogue or a 2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine analogue.

10

30. The composition of claim 28, wherein said second agent is a sulfonamide inhibitor of dihydropteroate synthetase.

15

31. The composition of claim 12, wherein said second agent inhibits polyamine synthesis.

20

32. The composition of claim 31, wherein said second agent is  $\alpha$ -methylornithine, 1,3-diaminopropan-2-ol, DFMO or DFMA.

25

33. The composition of claim 31, wherein said second agent inhibits S-adenosylmethionine decarboxylase (SAM-DC).

30

34. The composition of claim 33, wherein said second agent is AMA, MHZPA, MAOEA, AbeAdo, AdoMac, MGBG, CGP-39'937 or CGP-33'829.

35

35. The composition of claim 31, wherein said second agent inhibits ornithine decarboxylase (ODC).

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36. The composition of claim 31, wherein said second agent inhibits arginine decarboxylase (ADC).

5

37. The composition of claim 9-11, wherein said second agent inhibits RNA maturation.

10

38. The composition of claim 37, wherein said second agent is 5-azacitidine, cordycepin or toyocamycin.

15

39. The composition of any preceding claim, comprising between two and about five distinct antimicrobial agents in combination with said second agent.

20

40. The composition of any preceding claim, comprising said antimicrobial agent in combination with between two and about ten distinct second agents.

25

41. The composition of any preceding claim, comprising an MLS antibiotic in combination with a second agent and a non-MLS antibiotic.

30

42. The composition of any preceding claim, wherein said composition is dispersed in a pharmaceutically acceptable medium.

35

43. The composition of claim 42, wherein said pharmaceutically acceptable composition is suitable for human administration.

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44. The composition of claim 42, wherein said pharmaceutically acceptable medium is suitable for oral administration.

5

45. The composition of claim 42, wherein said pharmaceutically acceptable medium is suitable for parenteral administration.

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46. The composition of claim 42, wherein said pharmaceutically acceptable medium is suitable for topical administration.

15

47. The composition of claim 42, wherein said pharmaceutically acceptable medium is suitable for nasal administration.

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48. The composition of claim 42, wherein said pharmaceutically acceptable medium is suitable for ophthalmic administration.

25

49. The composition of claim 42, wherein said pharmaceutically acceptable medium is a liposomal formulation.

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50. An antimicrobial composition according to any preceding claim, for use in reducing the resistance of a microorganism to an antimicrobial agent.

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51. An antimicrobial composition according to any preceding claim, for use in reducing the resistance of a bacterium to an antibiotic.

5

52. An antimicrobial composition according to any preceding claim, for use in reducing the resistance of an MLS-susceptible bacterium to an MLS antibiotic.

10

53. An antimicrobial composition according to any preceding claim, for use in inhibiting RNA methylation or maturation in a microorganism.

15

54. An antimicrobial composition according to any preceding claim, for use in inhibiting RNA methylation or maturation in a bacterium.

20

55. An antimicrobial composition according to any preceding claim, for use in inhibiting RNA methylation in a microorganism or bacterium.

25

56. An antimicrobial composition according to any preceding claim, for use in inhibiting RNA maturation in a microorganism or bacterium.

30

57. An antimicrobial composition according to any preceding claim, for use in killing a microorganism.

35

58. An antimicrobial composition according to any preceding claim, for use in killing a bacterium.

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59. An antimicrobial composition according to any preceding claim, for use in killing an MLS-susceptible bacterium.

5

60. An antimicrobial composition according to any preceding claim, for use in reducing the resistance of a microorganism within an animal to an antimicrobial agent wherein the composition is administered to the animal in an amount effective to reduce the resistance of the microorganism to the antimicrobial agent.

61. An antimicrobial composition according to any preceding claim, for use in killing a microorganism within an animal wherein the composition is administered to the animal in an amount effective to kill the microorganism.

20

62. An antimicrobial composition according to any preceding claim, for use in treating a microbial infection wherein the composition is administered to an animal with a microbial infection in an amount effective to reduce said infection.

63. An antimicrobial composition according to any preceding claim, for use in treating a bacterial infection wherein the composition is administered to an animal with a bacterial infection in an amount effective to reduce said infection.

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64. An antimicrobial composition according to any preceding claim, for use in treating a human subject with a microbial or bacterial infection wherein the composition is administered to the human subject in an amount effective to reduce said infection.

65. Use of a composition according to any preceding claim in the manufacture of a medicament for treating a microbial infection in an animal wherein the medicament is administered to said animal in an amount effective to reduce said microbial infection.

66. Use of an agent that inhibits methylation in a microorganism in the manufacture of a medicament for treating a microbial infection in an animal wherein the medicament is administered to said animal in an amount effective to inhibit RNA methylation or maturation in a microorganism.

67. A use according to claim 66, wherein said agent is formulated into a medicament for administration to an animal prior to administration of an anti-microbial agent.

68. A use according to claim 66, wherein said agent is formulated into a medicament for administration to an animal subsequent to administration of an anti-microbial agent.

69. A use according to claim 66, wherein said agent is formulated into a medicament for administration to an

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animal simultaneously with administration of an anti-microbial agent.

5     70. A use according to claim 66, wherein said agent is formulated into a single medicament in combination with an anti-microbial agent.

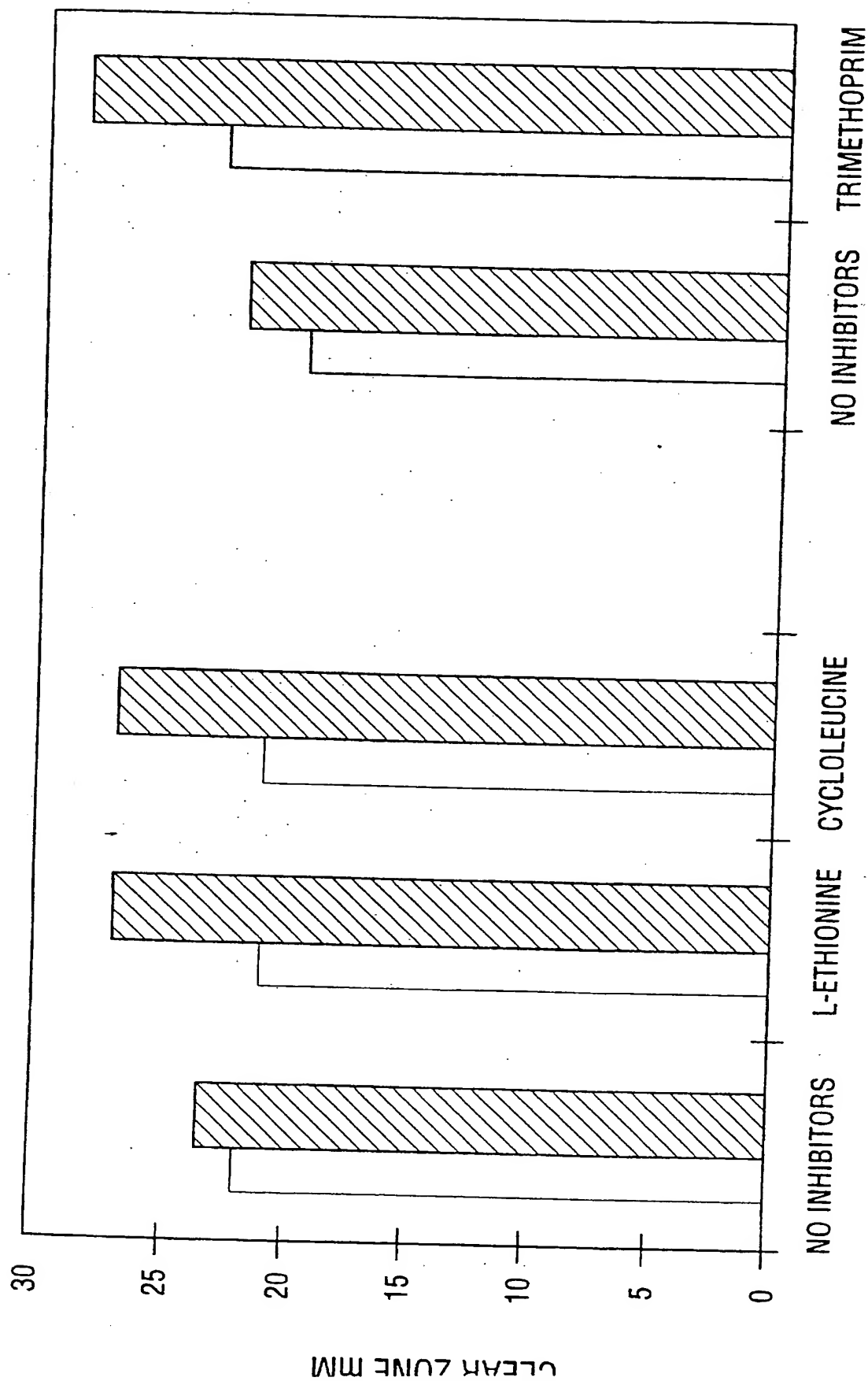
10    71. A kit comprising, in suitable container means, a pharmaceutical formulation of at least one antimicrobial agent and a pharmaceutical formulation of at least one microbial methylation inhibitor.

15    72. The kit of claim 71, wherein said antimicrobial agent and said methylation inhibitor are present within a single container means.

20    73. The kit of claim 71, wherein said antimicrobial agent and said methylation inhibitor are present within distinct container means.



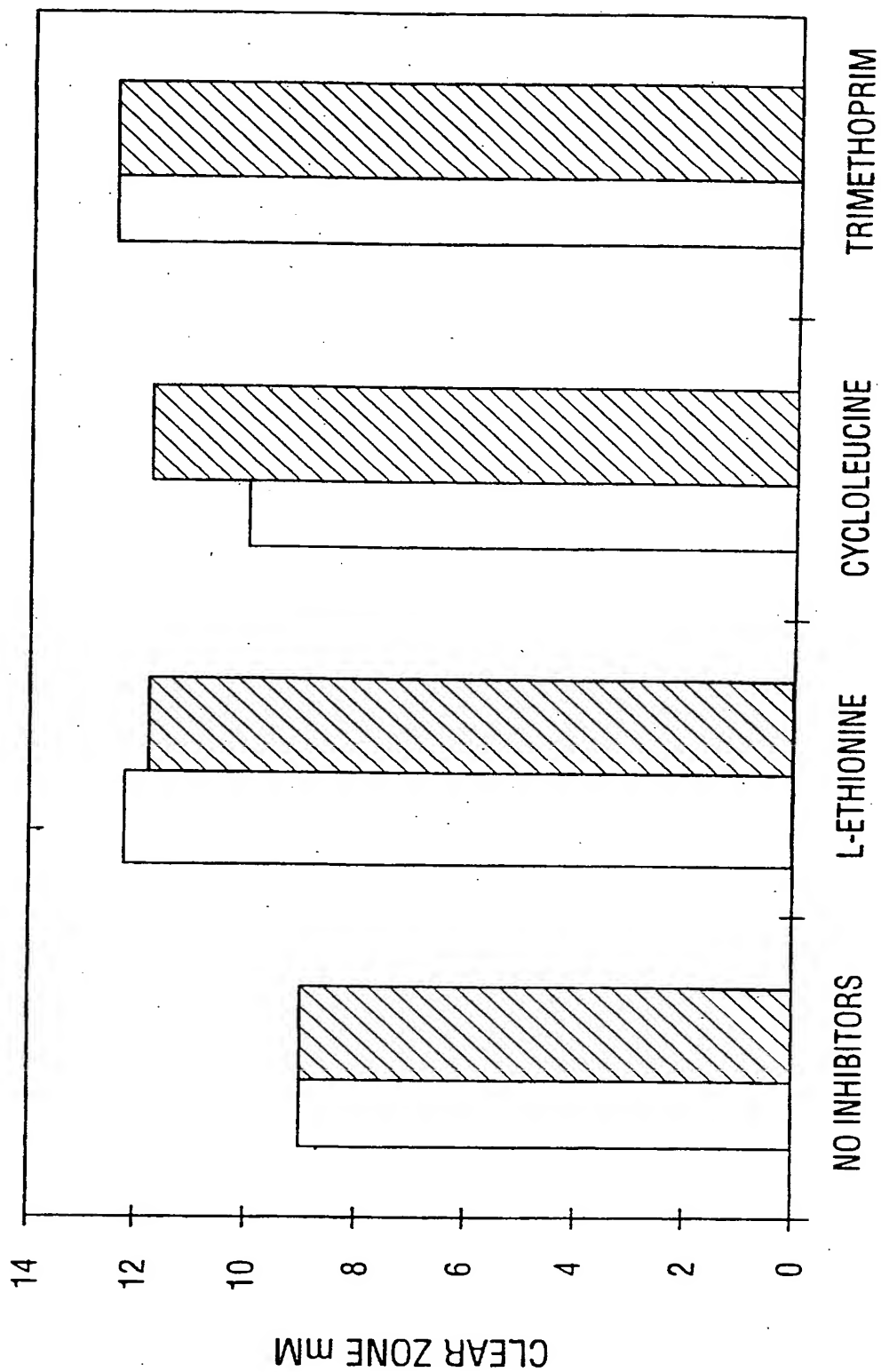
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INHIBITORS

FIG. 1

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PRE-MIXED INHIBITORS

FIG. 2

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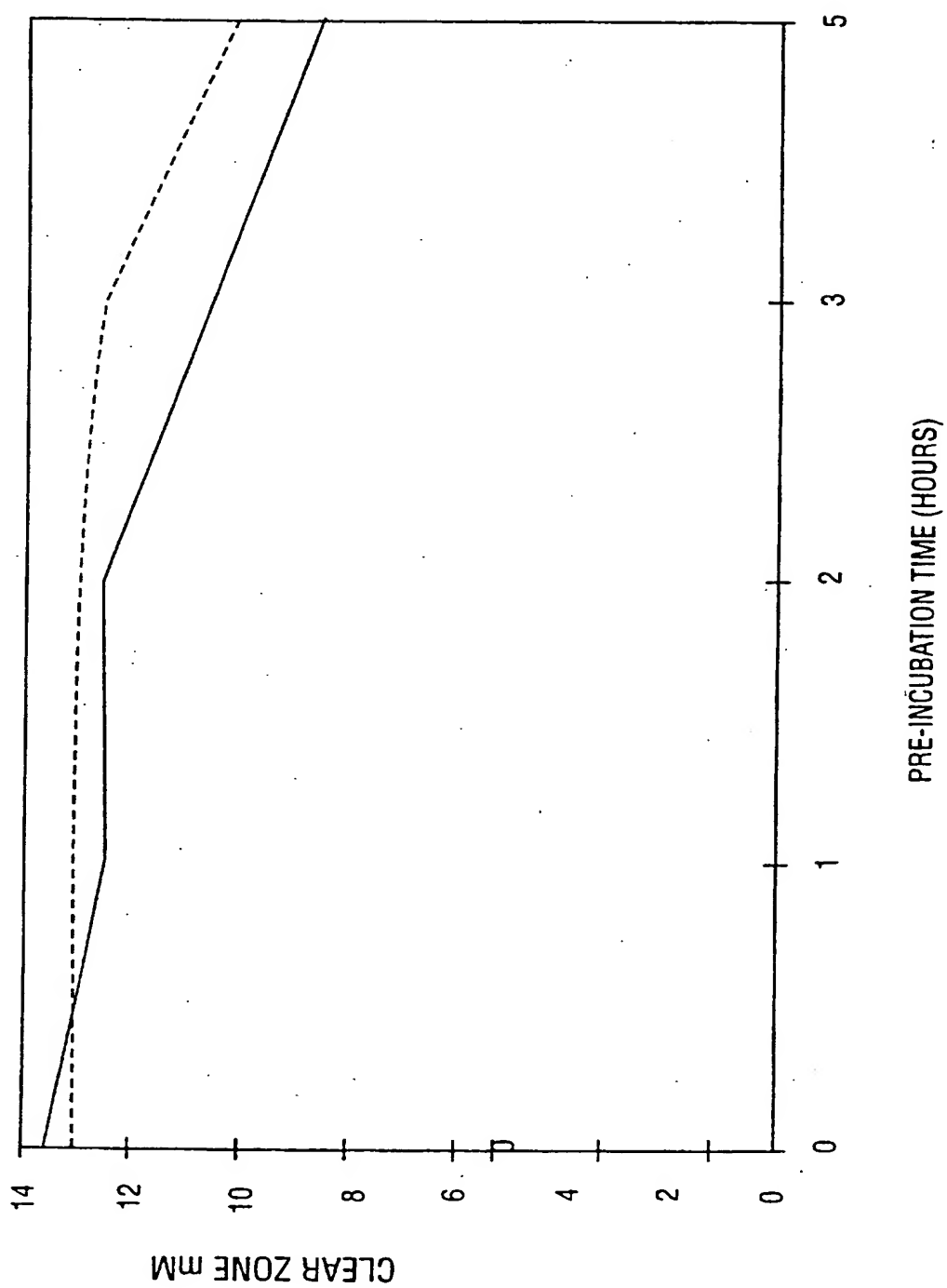


FIG. 3

# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US 95/16677

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K45/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 052 705 (BERNSTEIN JOEL E) 2 June 1982 see claims 1,2	1-6,9-14
X	J ANTIBIOT (TOKYO), 39 (9). 1986. 1291-1297., XP000571520 HASOBE M ET AL: "THE SYNERGISM OF NUCLEOSIDE ANTIBIOTICS COMBINED WITH GUANINE-7-N-OXIDE AGAINST A RHABDOVIRUS INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS" see page 1296, paragraph 4-7	1,9-18
X	PRESSE MED., 1994, 23/26 (1195-1198), FRANCE, XP000571949 TOUZE J.E.: "New therapeutic approaches to anti-parasite treatments" see abstract	1,9-12, 28

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- \*&\* document member of the same patent family

Date of the actual completion of the international search

29 May 1996

Date of mailing of the international search report

06.06.96

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# INTERNATIONAL SEARCH REPORT

Internatic Application No  
PCT/US 95/16677

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>J. INFECT. DIS., 1987, 155/5 (1027-2032),  USA, XP000571952  KOVACS J.A. ET AL: "Potent effect of  trimetrexate, a lipid-soluble antifolate,  on Toxoplasma gondii"  see abstract</p> <p>-----</p>	1,9-12, 28

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/16677

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-73  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please see annex!

3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

### Education on patient family members

PCT/US 95/16677

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0052705	02-06-82	US-A- 4505896	19-03-85
		AU-B- 7554481	03-06-82
		CA-A- 1176986	30-10-84
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